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The Regulation of Endometrial Repair and its Impact on Heavy Menstrual Bleeding

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Abstract

Introduction: The human endometrium has a remarkable capacity for efficient cyclical repair following the inflammatory process of menstruation. Defective post-menstrual repair may contribute to the common complaint of heavy menstrual bleeding (HMB). The mechanisms and factors involved in endometrial repair are still to be fully elucidated. Endometrial function is governed by the ovarian hormones and pre-menstrually progesterone levels decline as the corpus luteum regresses. Consequently, the synthesis of prostaglandins (PG) is increased, namely PGE_2 and the potent vasoconstrictor $PGF_{2\alpha}$. Subsequent vasoconstriction of endometrial spiral arterioles is believed to result in a transient hypoxic episode in the upper endometrial layer.

Therefore, the aims of this thesis were to determine (i) the endometrial expression of putative repair factors across the menstrual cycle (ii) the regulation of these factors by hypoxia, PGE_2 and $PGF_{2\alpha}$ (ii) the role of hypoxia inducible factor (HIF)-1α in endometrial repair and (iii) differences in endometrium from women with objectively measured HMB (>80ml) and normal controls (<80ml).

Methods/Results: Putative repair factors, with known angiogenic, mitogenic and proliferative functions, were identified in human endometrial samples by quantitative reverse transcription PCR and immunohistochemistry. Interleulin-8 (IL-8), vascular endothelial growth factor (VEGF), adrenomedullin (AM), connective tissue growth factor (CTGF) and endothelin-1 (ET-1) were all maximally expressed during the menstrual and/or proliferative phases of the cycle, consistent with the onset of endometrial repair. Endometrial cells and tissue explants treated with 100nM $PGE_2/F_{2\alpha}$ and/or hypoxia (0.5% O_2) revealed up-regulation of IL-8, VEGF, AM and CTGF. An *in vitro* progesterone antagonism model revealed that progesterone withdrawal, hypoxia and prostaglandins are all necessary for significant increases in repair factor expression in endometrial tissue. HIF-1α was detected in human endometrium but exclusively in the late-secretory and menstrual phases. Using short-hairpin RNA against HIF-1α, it was determined that hypoxia up-regulated these factors via HIF-1α, whereas $PGF_{2\alpha}$ acted in a HIF-1α independent manner to increase

repair factor expression. Finally, whole genome array analysis was performed on menstrual endometrium from women with objectively measured heavy and normal menstrual bleeding to provide an unbiased comparison of gene expression. 259 transcripts displayed significant changes between the two groups. Five candidate genes were validated using Q-RT-PCR. Bioinformatic analysis of the differentially expressed gene set identified bioprocesses that included positive regulation of biological and cellular processes, leukocyte differentiation, regulation of apoptosis and response to stress/hypoxia. The presence of HIF-1α protein was examined in menstrual endometrial tissue nuclear protein extracts by Western blot, revealing significantly decreased levels in women with HMB versus normal controls. Furthermore, the mRNA expression of known target genes of HIF-1α (VEGF, CXCR4) was also significantly decreased in these women. The functional impact of endometrial HIF-1α was assessed using an *in vitro* angiogenic assay. Silencing of HIF-1α in endometrial cells significantly reduced the angiogenic potential of culture supernatants when compared to untransfected cells or cells transfected with a scrambled sequence.

Conclusions: Repair factors are significantly increased in the human endometrium following the onset of menstruation. Progesterone withdrawal, hypoxia via HIF-1α and prostaglandins appear necessary for the regulation of these factors at this time. Menstrual endometrium displays significant differences in gene expression and HIF-1α protein levels between women with HMB and normal controls. The findings of this thesis contribute to the existing literature on both the physiological process of endometrial repair and the pathogenesis of HMB. Extension of this work may allow the identification of novel therapeutic targets for the treatment of this common, debilitating condition.

Declaration

In accordance with the requirements of the University of Edinburgh I hereby declare that the studies undertaken in this thesis were the unaided work of the author, except where due acknowledgement is made.

The work described herein has not previously been submitted or accepted for another degree or professional qualification.

Dr Jacqueline Maybin

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Abbreviations

11βHSD	11β hydroxysteroid dehydrogenase
ABC	avidin biotin peroxidase detection system
ACTG2	actin-gamma-2
AM	adrenomedullin
Ang-2	angiopoietin-2
ANOVA	analysis of variance
ARNT	aryl hydrocarbon receptor nuclear translocator (HIF-1β)
bHLH	basic helix-loop-helix
BSA	bovine serum albumin
BV	biliverdin
CBP	CREB binding protein
cDNA	complementary DNA
CLR	calcitonin receptor like receptor
CO	carbon monoxide
COX 1/2	cyclo-oxygenase-1/2
CTGF	connective tissue growth factor
CXCR4	chemokine (C-X-C motif) receptor 4
E2	oestradiol
EC	echinomycin
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
EP2S	ishikawa cell line stably transfected with the prostaglandin E receptor type 2
EP300	E1A binding protein p300
EPAS	endothelial PAS domain protein 1 (HIF-2α)
ER	ostrogen receptor
ERα/β	oestrogen receptor α/β
ESM1	endothelial cell specific molecule 1
ESR1	oestrogen receptor 1

ET-1	endothelin-1
FPS	ishikawa cell line stably transfected with the prostaglandin F2 alpha receptor
FSH	follicle stimulating hormone
GE	glandular epithelial cells
HES	human primary endometrial stromal cells
HEY-1	Hairy and enhancer of split-related protein 1
HIF-1α/β	hypoxia inducible factor-1α/β
HIF-2α	hypoxia inducible factor-2α
HMB	heavy menstrual bleeding (>80ml)
HO-1	heme-oxygenase-1
HRP	horseradish peroxidase
IDH1	isocitrate dehydrogenase 1
IK	ishikawa endometrial epithelial cell line
IL-1β	interleukin-1β
IL-8	interleukin 8
I-R	ischaemia-reperfusion
LH	luteinising hormone
LMP	the first day of the last menstrual period
Lv	blood vessel length density
Lv/Nv	mean vessel length per branch point
MBL	menstrual blood loss
MMP	matrix metalloproteinase
NBF	neutral buffered formalin
NFkB	nuclear factor kappa B
NMB	normal menstrual bleeding (<80ml)
Nv	branch point density
P	progesterone
PAI	plasminogen activator inhibitor
PAS	Per-ARNT-Sim
PBS	phosphate buffered saline
PG	prostaglandin

PHD	Prolyl hydroxylase
PNKD	Paroxysmal nonkinesigenic dyskinesia
PTGS2	prostaglandin-endoperoxide synthesase 2 or cyclooxygenase-2
Q-RT-PCR	quantitative reverse transcriptase polymerase chain reaction
RAMP	receptor activity modulating protein
RNA	ribosomal nucleic acid
SE	surface epithelial cells
ShRNA	short hairpain RNA
SiRNA	short interfering RNA
SMAD3	SMAD family member 3
St	stromal compartment
TAD	transactivation domain
TBST	tris buffered saline with Tween
TGFβ	transforming growth factor-β
TNFα	tumour necrosis factor α
t-PA	tissue plasminogen activator
u-PA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor

Publications

Original research papers

1. **Maybin JA,** Jabbour HN, Hirani N, Critchley HOD. A Novel Role for Hypoxia and Prostaglandin E_2 in the Regulation of IL-8 during Endometrial Repair. *Am J Pathol*. 2011 Mar; 178(3):1245-56.
2. **Maybin JA,** Battersby S, Hirani N, Nikitenko L, Critchley HOD, Jabbour HN. The Expression and Regulation of Adrenomedullin in the Human Endometrium: a candidate for endometrial repair**.** *Endocrinol*. 2011 July: 152(7):2845-56.
3. **Maybin JA**, Hirani N, Brown P, Jabbour HN, Critchley HOD. The Regulation of Vascular Endothelial Growth Factor by Hypoxia and Prostaglandin $F_{2\alpha}$ during Human Endometrial Repair. *J Clin Endocrinol Metab*. 2011 Aug; 96(8):2475-83.

Review articles

1. **Maybin JA,** Critchley HOD. Repair and Regeneration of the Human Endometrium. *Expert Rev Obst Gynecol***.** 2009; 4(3): 283-98.
2. **Maybin JA,** Critchley HOD, Jabbour HN. Inflammatory Pathways in Endometrial Disorders. *Mol. Cell. Endocrinol.* 2011 15; 335(1):42-51.
3. **Maybin JA**, Critchley HOD. Progesterone: a pivotal hormone at menstruation**.** . *Ann N Y Acad Sci*. 2011; 1221: 88-97.
4. Critchley HO, **Maybin JA.** Molecular and cellular causes of abnormal uterine bleeding of endometrial origin. Seminars in Reproductive Medicine, 2011: in press.

Copies of these publications can be found in Appendices 6 and 7.

Presentations

Oral presentations

1. **Maybin JA**, Jabbour HN, Hirani N, Critchley HOD. Hypoxia: an Initiator of Tissue Remodelling at Menstruation. Blair Bell Research Competition Meeting, London. November 2009. (Oral presentation prize).
2. **Maybin JA**, Battersby S, Hirani N, Critchley HOD, Jabbour HN. The expression and regulation of endometrial adrenomedullin: a novel candidate for endometrial repair. Society for Reproduction and Fertility, Nottingham, July 2010. (Oral presentation prize).
3. **Maybin JA**, Battersby S, Hirani N, Jabbour HN, Critchley HOD. A novel role for hypoxia and prostaglandin F2α in the regulation of adrenomedullin for endometrial repair. ESHRE, Rome, June 2010.
4. **Maybin JA**, Barcroft J, Hirani N, Jabbour HN, Critchley HOD. Endometrial expression of connective tissue growth factor (CTGF) and its dysregulation in women with heavy menstrual bleeding. British International Congress of Obstetrics and Gynaecology. Belfast, June 2010.
5. **Maybin JA**, Hirani N, Jabbour HN, Critchley HOD. A novel role for hypoxia in the regulation of IL-8 during endometrial repair. Society for Gynecological Investigation. Orlando, Florida, March 2010.
6. **Maybin JA,** Hirani N, Marshall E, Jabbour H, Critchley HOD. Hypoxia inducible factor-1 in the human endometrium and its impact on heavy menstrual bleeding. Society for Gynecological Investigation. Miami, Florida, March 2011.

Poster presentations

1. **Maybin JA,** Hirani N, Marshall E, Jabbour H, Critchley HOD. Hypoxia inducible factor-1 and the regulation of human endometrial repair following menstruation. Medical Research Society, London, February 2011.
2. **Maybin JA,** Jabbour HN, Hirani N, Critchley HOD. Hypoxic regulation of vascular endothelial growth factor during endometrial repair. Medical Research Society, London, February 2010.
3. **Maybin JA,** Jabbour HN, Hirani N, Critchley HOD. The Role of PGE_2 and Hypoxia in the Up-regulation of CTGF and ET-1 in the human endometrium. Society for Gynecological Investigation, Glasgow. March 2009.
4. **Maybin JA**, Jabbour HN, Hirani N, Critchley HOD. The Role of PGE_2 and Hypoxia in the Up-regulation of CXCL8 (IL-8) and Endothelin-1 (ET-1) in the human endometrium. Scottish Society for Experimental Medicine, Edinburgh. Nov 2008.

Prizes

1. MRC/Guardian Max Perutz Science Writing Prize, 2009. Copy in appendix 8.
2. Blair Bell Research Society (RCOG) Oral Presentation Prize, 2009
3. Society for Reproduction and Fertility Oral Presentation Prize, 2010
4. Society for Endocrinology post-graduate essay writing runner up prize, 2010. Copy in appendix 8.

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1. Literature Review

1.1 The Human Endometrium and Menstrual Cycle

The human endometrium displays a remarkable capacity for repair. Throughout the reproductive years of a woman's life it is cyclically subjected to the inflammation and tissue destruction of menstruation. However, each cycle the endometrium can fully repair without loss of function or scarring. The mechanisms involved in this efficient endometrial repair have still to be fully defined. Aberrations may lead to common gynaecological pathologies, such as heavy menstrual bleeding and dysmenorrhoea (painful menstruation). This thesis focuses on the delineation of potential regulatory pathways involved in endometrial repair and their contribution to heavy menstrual bleeding (HMB).

1.1.1 The ovarian cycle

The cyclical shedding, repair and regeneration of the human endometrium is driven by the ovarian sex steroids (Johnson and Everitt, 2000) (Figure 1). Neurones in the hypothalamus secrete gonadotrophin releasing hormone in a pulsatile fashion. This causes the synthesis and release of lutenising hormone (LH) and follicle stimulating hormone (FSH) from the pituitary to regulate ovarian hormone production. During menstruation, increased FSH secretion stimulates a cohort of antral follicles to undergo preovulatory development.

As the follicles grow they secrete increasing amounts of oestradiol, which negatively feeds back to the pituitary to lower FSH secretion. As smaller follicles require higher levels of FSH to survive than larger ones, a dominant follicle emerges and the smaller follicles undergo atresia. When the follicle is fully mature the high concentrations of oestradiol have a positive feedback effect at the hypothalamus and pituitary to induce an LH surge and ovulation. After ovulation, the remaining granulosa and theca cells form the corpus luteum. The corpus luteum produces the high levels of progesterone that dominate the secretory phase of the cycle, as well as producing oestradiol (E_2) and inhibin A. These hormones maintain negative feedback on the pituitary. In the absence of pregnancy, the corpus luteum becomes less sensitive to LH, and luteolysis occurs. This results in a dramatic decrease in progesterone production (progesterone withdrawal). This decline in sex steroid

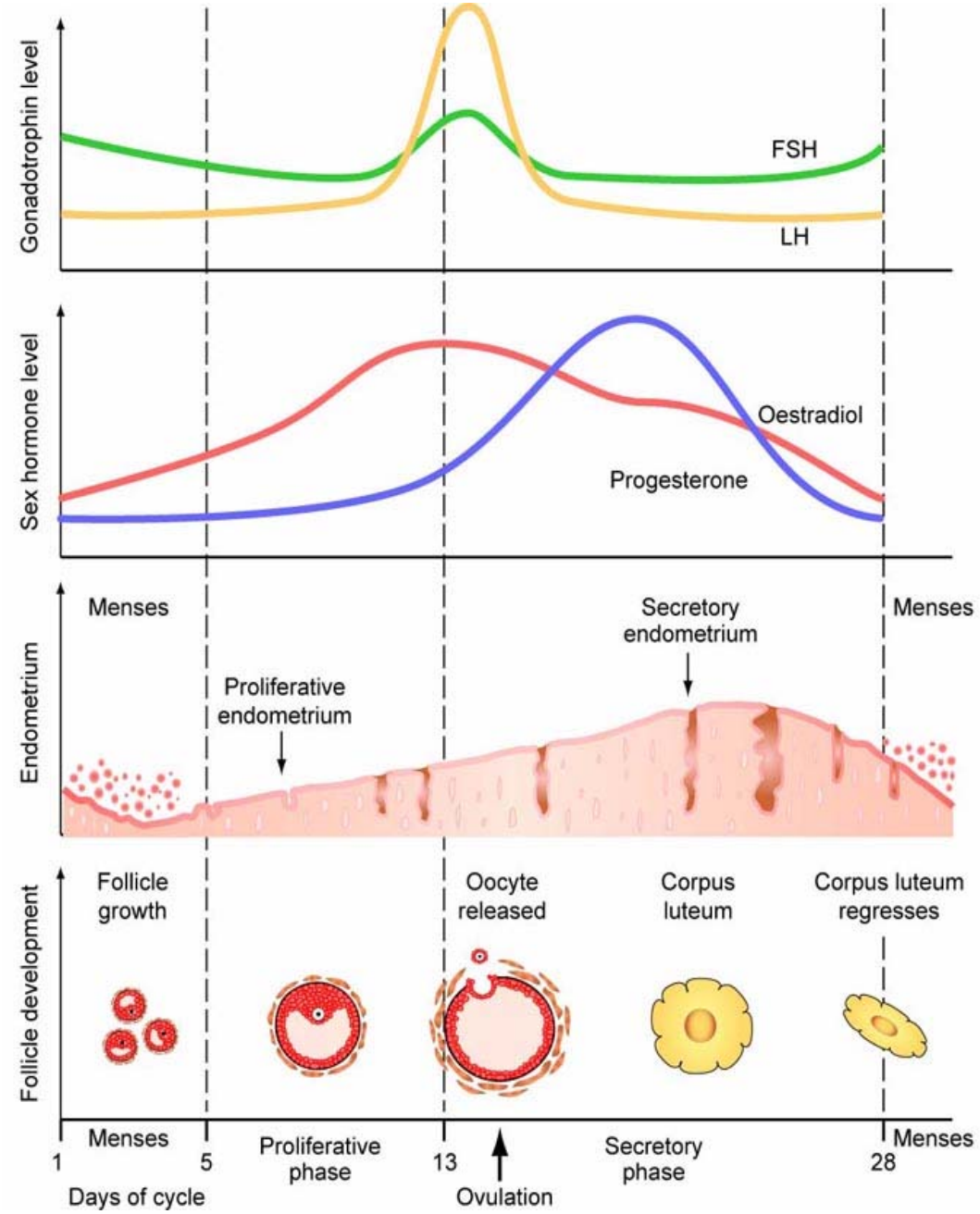


Figure 1. **The human menstrual cycle**. Gonadotrophin release governs sex hormone production by the ovaries, leading to local endometrial changes across the menstrual cycle (**FSH** follicle stimulating hormone, **LH** lutenising hormone).

production reduces negative feedback on the pituitary, allowing the increase in FSH production required to stimulate the next cycle.

1.1.2 Endometrial histology

The fluctuations in ovarian E_2 and progesterone production are reflected by well documented morphological changes in the endometrium (Noyes et al., 1950, Buckley and Fox, 1989). The human endometrium is composed of two distinct compartments; the basal layer and an upper functional layer (Figure 2). It is the functional layer that is shed during menstruation. Traditionally, regeneration of the endometrium was thought to occur from the remaining basal layer but recent investigation of laser capture microdissected basal and functional menstrual endometrium suggested the shed functional layer also contributes to this process (Gaide Chevronnay et al., 2009).

The human endometrium is a dynamic tissue consisting of various cell types, including the luminal and glandular epithelial cells and a stromal compartment. The stromal compartment contains fibroblast-like cells as well as the vascular and lymphatic systems. Endometrial vessels become heavily coiled in the second half of the menstrual cycle and consist of endothelial cells and surrounding pericytes. These specialised spiral arterioles differ from other arterioles by the absence of an internal elastic lamina (Robertson and Manning, 1974). They arise from radial arteries at the endometrial/myometrial border and take a tortuous course towards the endometrial luminal surface. Endometrial lymphatic vessels are much less well described but are thought to be sparsely distributed in the functional layer, with larger lymphatic vessels in the basal layer, often closely associated with spiral arterioles (Donoghue et al., 2007).

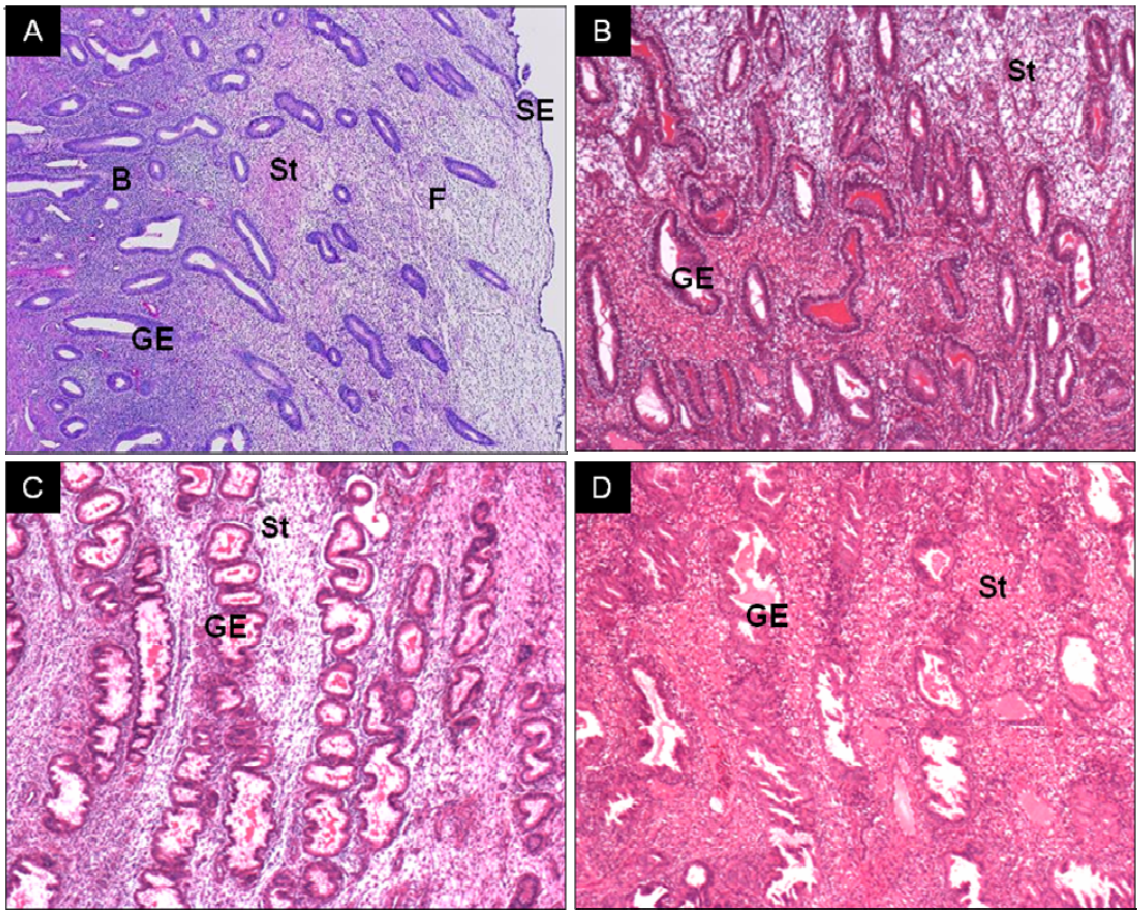


Figure 2. **Endometrial histological changes across the menstrual cycle**. (A) Proliferative, (B) Early secretory, (C) Mid Secretory and (D) Late Secretory phase endometrium. B: basal layer, F: functional layer, GE: glandular epithelial cells, SE: surface epithelial cells, St: stromal cell compartment.

1.1.3 Menstrual phase

The menstrual phase of the cycle commences on day 1 following withdrawal of ovarian steroids and lasts until day 5-7. Detailed study of early endometrial growth in the human endometrium has been performed using scanning electron microscopy (Ludwig and Spornitz, 1991). After shedding of the functional layer at menstruation, the endometrium has a ragged and torn surface with gland openings and a lack of epithelial covering. Regrowth of the luminal epithelium occurs initially, prior to stromal expansion. This begins on day 2, during active bleeding, and full coverage of the lumen is usually achieved by day 6. A more recent hysteroscopic, histological and scanning electron microscopy study identified that the endometrium is shed in a piecemeal fashion (Garry et al., 2009). Furthermore, this study identified that the endometrial repair process commences in delineated areas while shedding occurs simultaneously in adjacent areas. Recent laser capture microdissection techniques have identified that both the stromal and epithelial cell compartments have an active role in this repair process (Gaide Chevronnay et al., 2010).

1.1.4 Proliferative phase

The proliferative phase of the menstrual cycle follows menstruation and lasts until ovulation (day 14 of a 28 day cycle). E_2 is the dominant hormone of this phase and its production increases as the dominant follicle develops. The early proliferative phase lasts from approximately day 4 to 7 and is characterised by regeneration of the newly repaired endometrium after menstruation. During the mid and late proliferative phase (day 8 to 10 and day 11 to 13 respectively) there is continued growth and development of glands, stroma and vasculature. Histologically, proliferative glands are narrow and straight and the stromal compartment is compact (Noyes et al., 1950) (Figure 2A).

1.1.5 Secretory phase

Following ovulation, luteal production of progesterone dominates the endometrial endocrine environment and the secretory phase begins. During this phase emphasis switches from proliferation to differentiation and it is divided into three stages (i) early: day 14 to 18, (ii) mid: day 19 to 23 and (iii) late: day 24 to 28. The histological

appearance of the secretory endometrium has been described (Noyes et al., 1950, Buckley and Fox, 1989). After ovulation, sub-nuclear vacuoles appear in the glandular epithelium and the glands become tortuous (Figure 2B). By the mid secretory phase secretory activity of the glands is apparent, causing stromal oedema (Figure 2C). During this phase the endometrium is receptive to implantation should fertilisation occur. The late secretory phase is characterised by the maturation and differentiation of the spiral arterioles. The perivascular cell number increases with some cells undergoing decidualisation (Figure 2D). If pregnancy does not occur, glandular secretion declines and the endometrium becomes dehydrated, giving the glands a saw-toothed appearance. There is an influx of leukocytes during this phase (discussed in detail in section 1.2.1). The withdrawal of ovarian steroids during this phase results in tissue necrosis and menstruation.

1.2 Menstruation as an inflammatory process

Menstruation displays many of the classic hallmarks of inflammation, including tissue oedema and influx of immune cells. Withdrawal of steroid hormones, progesterone in particular, during the late secretory phase triggers a cascade of molecular and cellular interactions, culminating in menstruation (Finn and Pope, 1986). Breakdown of the extracellular matrix is mediated by key enzymes; the matrix metalloproteinases (MMPs) (Marbaix et al., 1996, Salamonsen and Woolley, 1999). These enzymes have the ability to degrade all components of the extracellular matrix (ECM) and are up-regulated at the time of menstruation as a result of progesterone withdrawal (Vassilev et al., 2005). However, MMPs in the perimenstrual phase are limited to the functional layer, suggesting a more local regulation. Leukocytes express MMPs and have the potential to stimulate MMP production from adjacent cells (Jabbour et al., 2006) making them potential candidates in the regulation of endometrial MMPs.

1.2.1 Leukocytes

Following progesterone withdrawal there is a dramatic rise in the endometrial leukocyte population. Neutrophil numbers are negligible throughout the cycle but increase perimenstrually to comprise 6-15% of the total cell number (Salamonsen and Lathbury, 2000). The most prevalent leukocyte is the uterine NK (uNK) cell (King, 2000), comprising 70% of endometrial leukocytes. These uNK cells are $CD56^{bright}$, CD16-, CD3- and are phenotypically different from peripheral blood NK cells (Koopman et al., 2003). uNK cells increase in number during the mid-luteal phase and are located close to the endometrial glands and spiral arteries (Dosiou and Giudice, 2005). Their location suggests they have a role in vascular remodelling and the lack of spiral arteriole modification seen in mice deficient in uNK cells supports this hypothesis (Ashkar et al., 2003, Greenwood et al., 2000). uNK cells produce a wide range of regulatory cytokines, indicating a role in initiation of cellular and humoral responses. Macrophages also increase in number throughout the secretory phase to reach maximal numbers perimenstrually (Bonatz et al., 1992, Critchley et al., 1999). These cells are involved in tissue remodelling and debris removal. The increase in leukocyte numbers during the perimenstrual phase implicates up-

regulation due to progesterone withdrawal. However, the inability to identify the PR receptor on macrophages or uNK cells (King et al., 1996, Stewart et al., 1998, Henderson et al., 2003) suggests an indirect mechanism of hormonal regulation. Paracrine mediators are likely to control leukocyte regulation at the time of menstruation, with a range of chemokines emerging as potential candidates (Hannan and Salamonsen, 2007, Salamonsen and Lathbury, 2000).

1.2.2 Chemokines

Chemokines are chemotactic cytokines involved in leukocyte recruitment and activation. They have been implicated in numerous reproductive events, including menstruation and implantation (Dominguez et al., 2003). Chemokines are divided into four subclasses; C, CC, CXC and CX3C, depending on their amino-terminal cysteine motif. Progesterone withdrawal up-regulates a host of inflammatory mediators *in vivo*, including the chemokines CXCL8 (Interleukin-8) and CCL-2 (monocyte chemoattractant protein-1) (Critchley et al., 1999). Gene microarray analysis of endometrial biopsies from women given the progesterone receptor antagonist mifepristone in the mid secretory phase revealed that 571 genes displayed significant changes when compared to untreated controls (Catalano et al., 2007). These included increases in CXCL5, 12 and CX3CL1 in biopsies taken six hours after mifepristone administration, signifying regulation of chemokine production by progesterone withdrawal. Endometrial chemokines appear to be tightly regulated through post-translational modification by MMPs and other proteases, which convert precursor forms into active chemokines (Hannan and Salamonsen, 2007). Immunohistochemical studies have localised endometrial chemokine production to the glandular cells and decidualised stromal cells (Jones et al., 2004).

1.2.3 Prostaglandins

The enzyme involved in prostaglandin synthesis, COX-2, is also up-regulated by progesterone withdrawal (Critchley et al., 1999) (Figure 3). Subsequent increased synthesis of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the endometrium has important implications for menstruation (Baird et al., 1996). There are significant numbers of prostaglandin receptors in the perivascular compartment of the endometrium (Milne and Jabbour, 2003, Milne et al., 2001) signifying a

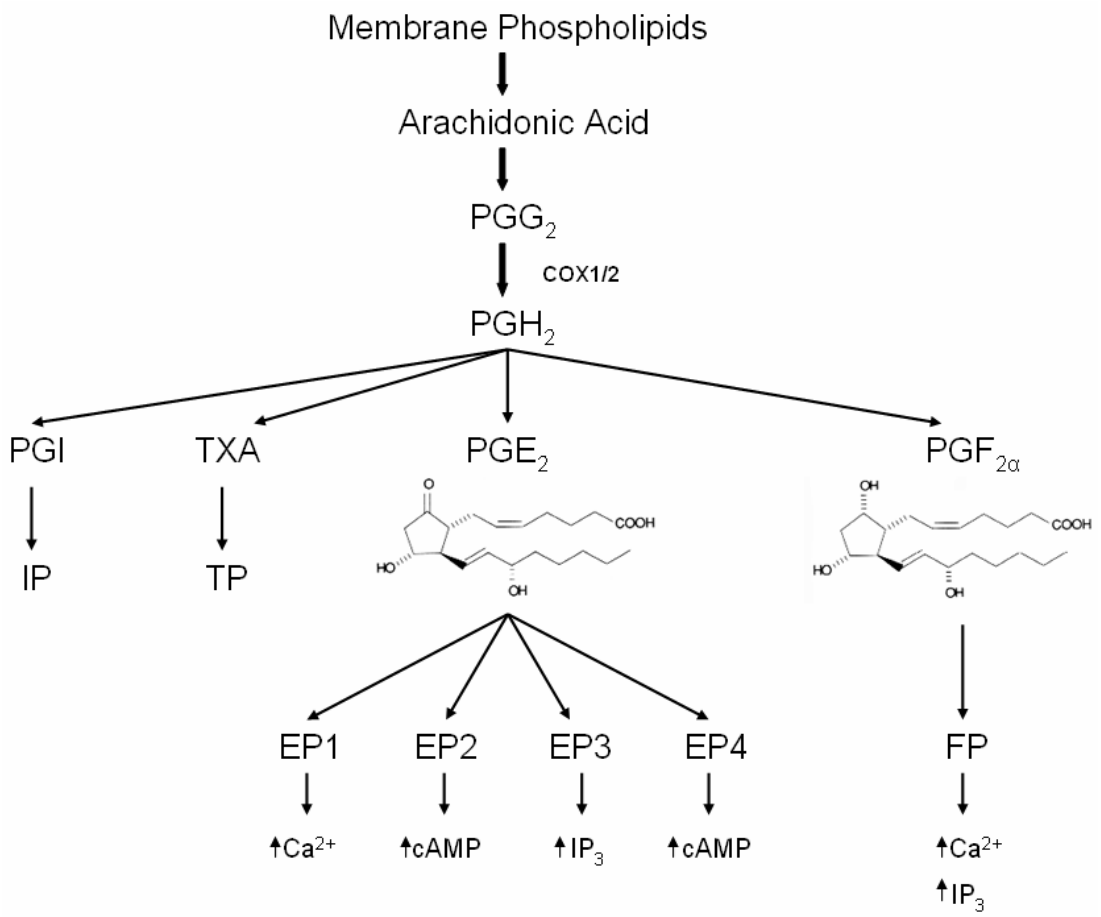


Figure 3. **Synthesis and signalling of prostaglandins** (PG). Cyclo-oxygenase 1/2 (COX-1/2), prostaglandin E receptors (EP), prostaglandin $F_{2\alpha}$ receptors (FP), thromboxane (TXA), thromboxane receptor (TP), prostacyclin (PGI), prostacyclin receptor (IP), cyclic adenosine monophosphate (cAMP), Inositol trisphosphate (IP_3).

functional role. PGE_2 is a potent vasodilator, leading to increased oedema and may contribute to pain at the time of menstruation (Williams, 1979). Administration of PGE_2 to healthy participants increased the diameter of cranial vessels and resulted in headache (Wienecke et al., 2009), demonstrating the pain inducing properties of this prostanoid. Interestingly, PGE_2 has been shown to act synergistically with CXCL8 to increase plasma leakage and neutrophil accumulation in the skin (Colditz, 1990). $PGF_{2\alpha}$ is also vasoactive, inducing myometrial contractions and vasoconstriction (Lundstrom, 1977).

1.2.4 Nuclear factor-κB

Nuclear factor-κB (NF-κB) is an inducible transcription factor known to increase a variety of inflammatory molecules. It consists of different sub-units, including p50, p65, cRel and Rel B. NF-κB is held captive in the cytoplasm by its inhibitor IκB until stimulated by an extracellular signal, e.g. IL-1, TNF-α. This results in phosphorylation of IκB and its degradation via an ubiquitin-proteosome pathway. NF-κB is then translocated to the nucleus and activates the transcription of target genes. NF-κB increases the transcription of a wide variety of genes, including cytokines (IL-1, IL-6), chemokines (CXCL8, CCL-2) and adhesion molecules (ICAM, VCAM) (Kayisli et al., 2004). Human endometrial biopsies have been shown to express components of the NF-κB pathway and there is evidence for activation of NF-κB during the perimenstrual phase (King et al., 2001). Progesterone has inhibitory effects on NF-κB activity, mediated by increasing IκB production or by competing with NF-κB for recognition sites on relevant genes (Kelly et al., 2001). In this way the steroid hormones modulate the endometrial inflammatory environment by suppressing NF-κB activity until menstruation is required.

1.3 The processes involved in endometrial repair

The human endometrium displays a remarkable capacity for repair following the florid inflammation associated with menstruation. This repair occurs with the absence of scarring and is so efficient that the endometrium regains full function in the subsequent cycle. The processes involved in endometrial repair have still to be fully delineated but are likely to be comparable to classic wound healing processes. These involve temporally overlapping phases of inflammation and its resolution, tissue formation, tissue remodelling and angiogenesis. The ability of the endometrium to regenerate in each menstrual cycle also raises the possibility of the presence of adult progenitor cells within the local endometrial environment (Gargett, 2007). While acknowledging this important aspect of endometrial repair, study of these controversial cells is beyond the scope of this thesis. Therefore, this section focuses on the classic wound healing processes described above. The regulation of endometrial repair is discussed subsequently (Section 1.4).

1.3.1 Inflammation

Although endometrial inflammation results in tissue destruction and menstruation, it is also likely to form a fundamental component of the repair process. Withdrawal of the anti-inflammatory ovarian steroid progesterone during the late secretory phase increases endometrial chemokine production (Critchley et al., 2003, Salamonsen and Woolley, 1999). Chemokines promote leukocyte adhesion and extravasation, resulting in endometrial accumulation of these cells in the perimenstrual endometrium (Salamonsen and Lathbury, 2000, Le et al., 2004). Leukocytes have an active role in the endometrial repair process. In the mouse model of menstruation, neutrophil depletion using the antibody RB6-8C5 affected endometrial breakdown and markedly delayed endometrial repair (Kaitu'u-Lino et al., 2007b). Macrophage depletion has also been shown to result in defective repair, as demonstrated in a guinea pig model of adult skin wounds (Leibovich and Ross, 1975) and in myocardial injury in mice (van Amerongen et al., 2007). The timing, number and morphology of inflammatory cells appear to be critical for efficient tissue repair. Persistence of leucocytes at a site of injury leads to ineffective wound healing and excess fibrosis (Wynn, 2008). Investigation of endometrial macrophage morphology

during the late secretory phase revealed lower levels of activation and adhesion molecules (CD69 and CD54) when compared to macrophages found in peritoneal fluid and may serve to limit the inflammatory response at menstruation (Eidukaite and Tamosiunas, 2004).

In addition to inflammatory cell influx, increased chemokine production in the perimenstrual endometrium may itself contribute to the endometrial repair process. Interleukin-8 (IL-8, CXCL8) is a CXC chemokine, best known for its role as a potent chemoattractant for neutrophils and T-cells (Larsen et al., 1989). In addition, it has been shown to induce mitogenesis of vascular smooth muscle cells (Yue et al., 1994) and to have a key role in angiogenesis *in vivo* (Koch et al., 1992). These processes are fundamental for endometrial shedding and repair. Previous studies have found IL-8 to be present in the human endometrium with an increase in IL-8 mRNA and protein during the late secretory phase (Arici et al., 1998, Milne et al., 1999). IL-8 interacts with two chemokine receptors CXCR1 and CXCR2; both have previously been shown to be expressed in the endometrium throughout the menstrual cycle (Mulayim et al., 2003).

1.3.2 Resolution of Inflammation

Similar processes may govern stimulation and resolution of inflammation, to induce a self-limiting inflammatory response at menstruation. The pro-inflammatory cytokine IL-1 has been shown to increase 11β hydroxysteroid dehydrogenase-1 (11βHSD-1) (Rae et al., 2004). This enzyme converts cortisone to the anti-inflammatory steroid cortisol. Endometrial 11βHSD-1 mRNA levels at menstruation have been demonstrated to be significantly increased, consistent with a role in resolution of endometrial inflammation (McDonald et al., 2006). Hence, local generation of glucocorticoids may prevent an excessive inflammatory response in the menstrual endometrium. Interestingly, women with heavy menstrual bleeding (HMB) were found to have significantly elevated levels of 11βHSD-2, which converts cortisol back to cortisone (Rae et al., 2009). Decreased cortisol levels and loss of its anti-inflammatory effects may prolong menses, contributing to heavy blood loss.

Another potentially important modulator of the inflammatory response in the endometrium is heme-oxygenase-1 (HO-1). As the rate limiting enzyme in the breakdown of heme, HO-1 has a protective role in times of tissue stress. In particular, HO-1 protects tissue from ischemia-reperfusion (I-R) injury, inflammation, hypoxia, haemorrhagic shock and heavy metal exposure (Farombi and Surh, 2006). In endothelial cells, HO-1 over-expression decreased levels of soluble Flt-1 and soluble endoglin (Cudmore et al., 2007). Both these mediators are increased in pre-eclampsia and their suppression by HO-1 suggests a protective role for HO-1 in pregnancy. Increased HO-1 also provides protection against liver damage induced by the chemical compounds acetaminophen and carbon tetrachloride (Farombi and Surh, 2006). HO-1 is highly inducible, by haemin and a range of non-haem factors including ultraviolet, heavy metals, hypoxia and nitric oxide (Tyrrell et al., 1993, Motterlini et al., 2000, Keyse and Tyrrell, 1989). Its induction results in the catabolism of haem to biliverdin (BV), carbon monoxide (CO) and free iron. These products mediate the cytoprotective effects of HO-1. BV is an endogenous antioxidant, as demonstrated in the rat liver model (Fondevila et al., 2004). When rat livers were subjected to I-R injury, adjuvant therapy with BV increased portal blood flow and decreased hepatocyte damage. After orthotopic liver transplantation, BV therapy increased animal survival from 50% to 90-100% (Fondevila et al., 2004). There was decreased infiltration of neutrophils and macrophages and a decrease in the number of anti-apoptotic molecules. CO production lead to vasodilation in the rat model via an increase in cyclic guanylate monophosphatase (cGMP) (Pannen et al., 1998). This stimulated relaxation of smooth muscle in vessel walls leads to vasodilation and increased clearance of free heme, limiting its toxic effects.

HO-1 has been identified in the human endometrium, localized to epithelial cells and macrophages (Yoshiki et al., 2001). Further studies are required to delineate the role of HO-1 in the endometrium as it has an attractive potential role in suppression of inflammation and initiation of endometrial repair. At the time of menstruation, tissue destruction and the associated bleeding results in high levels of haem in the uterine cavity. This hemorrhagic inflammation will theoretically stimulate HO-1 expression. Interestingly, administration of E_2 to rats following trauma-haemorrhage increased

the expression of HO-1 in the liver (Hsu et al., 2007). If E_2 has similar effects in the endometrium, it will provide a mechanism of endometrial protection following the hemorrhagic inflammation of menstruation. Effective resolution of inflammation post menstruation is crucial for endometrial repair. Intensive research into this aspect of endometrial function has potential transferable value for resolution of tissue injury elsewhere in the body.

1.3.3 Tissue formation

Detailed study of early endometrial growth using scanning electron microscopy of the human endometrium has been performed (Ludwig and Spornitz, 1991). After shedding of the functional layer at menstruation, the endometrium has a ragged and torn surface with gland openings and a lack of epithelial covering. Regrowth of the epithelium occurs first, prior to stromal expansion. Epithelial cells grow from the necks of the glands and spread to meet migrating cells from other glands, forming a new luminal surface. This begins on menstrual day 2 and full coverage of the lumen is achieved by day 6. The cellular and molecular mechanisms governing epithelial cell proliferation and migration after menstruation have not been fully elucidated. One hypothesis is the “free-edge” effect, where the absence of neighbouring cells at the wound margin acts as a growth signal (Heimark and Schwartz, 1985). Alternatively, examination of the mouse model of simulated menstruation has suggested that re-epithelialisation of the uterine surface arises from progenitor cells residing in the glandular epithelial cells (Kaitu'u-Lino et al., 2010). Examination of bromodeoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA) immunofluorescence in this model revealed that glandular cells proliferated selectively during repair and BrdU labelling remained constant. In contrast, there was no significant difference in luminal cell proliferation before and after endometrial breakdown and rapid dilution of BrdU was observed.

Growth factors released from stromal cells, epithelial cells, leukocytes and the ECM are likely to be important in endometrial repair. The ECM contains embedded cytokines and growth factors, (Mott and Werb, 2004) that may be released by MMP-mediated breakdown at menstruation. In addition to the known high levels of MMPs

at menstruation, MMP-7 mRNA is highly expressed during the early proliferative phase and remains abundant throughout the secretory phase (Vassilev et al., 2005). The active form of MMP-7 specifically binds to endometrial epithelial cells and exhibits resistance against tissue inhibitor of metalloproteinase-2 (Berton et al., 2007). MMP-7 null mice have demonstrated the importance of MMP-7 in re-epithelialisation (Dunsmore et al., 1998). These studies suggest that, alongside their ability to initiate menstruation, MMPs may have an active role in repair and remodeling of the post-menstrual endometrium.

The transforming growth factor-β superfamily is a group of pleiotropic growth factors with an important role in wound healing. TGF-β1 is a general regulator of cell proliferation, differentiation and extracellular matrix (ECM) repair. In human endometrial tissue, TGF-β1 mRNA and protein levels were reported to be maximal at menstruation (Gaide Chevronnay et al., 2008), suggesting a role in local repair mechanisms. There is evidence that MMP2 and 9 mobilise and activate TGF-β from bone matrix (Dallas et al., 2002) and a similar mechanism may be present in the perimenstrual endometrium. Culture of endometrial stromal cells with TGF-β1 *in vitro* resulted in significant suppression of progesterone receptor mRNA and attenuated protein levels, hence augmenting progesterone withdrawal. In addition, TGF-β1 significantly inhibited the expression of Dickkopf-1, a protein that inhibits Wnt signalling (Kane et al., 2008). The Wnt signalling cascade is thought to mediate uterine proliferation during the proliferative phase (Hou et al., 2004). Therefore, inhibition of a Wnt antagonist by TGF-β1 may increase endometrial repair.

Connective tissue growth factor (CTGF) is a multifunctional growth factor that is a member of a family of cysteine-rich secreted proteins. In adult mammals, CTGF is expressed at high levels during wound repair and at sites of connective tissue formation (Igarashi et al., 1993). Biological effects of CTGF include chemotaxis, differentiation, ECM production, angiogenesis, tumour growth, wound healing and fibrosis (Ivkovic et al., 2003, Frazier et al., 1996, Hishikawa et al., 2000, Shimo et al., 2001, Igarashi et al., 1993, Oemar et al., 1997). CTGF is therefore an attractive candidate for endometrial repair. It has been shown to be transcriptionally activated

by TGF-β1 and hypoxia (Higgins et al., 2004, Harlow et al., 2002, Brunner et al., 1991). CTGF has been detected in the human endometrium by immunohistochemistry and northern blot analysis (Uzumcu et al., 2000). Localisation revealed its presence in epithelial and endothelial cells in the proliferative and secretory phases, whereas stromal cells were only positively stained after decidualisation in the late secretory phase. No menstrual phase biopsies were included in this study but the presence of CTGF in the normal cycling endometrium suggests it may facilitate tissue repair and remodelling after menses.

1.3.4 Tissue remodelling

Following the rapid initial repair of the human endometrium, regrowth and regeneration takes place throughout the proliferative phase. Tissue recombination studies in the mouse model suggest that uterine epithelialisation is required for stromal responsiveness to ovarian steroids (Bigsby, 2002). This explains the sequence of re-epithelialisation preceding stromal expansion. Stromal cell mitoses first appear on day 5-6 of the human menstrual cycle, when E_2 levels are rising and the epithelial layer is completely healed (Ferenczy et al., 1979). Macrophages, fibroblasts and blood vessels are required for endometrial regrowth. Macrophages provide essential growth factors, fibroblasts replenish the ECM, and blood vessels supply oxygen and nutrients necessary for cell metabolism. Synthesis and remodelling of the ECM by fibroblasts is essential for wound healing. Fibroblasts differentiate into contractile phenotypes during the last phases of wound healing and increase their smooth muscle actin (SMA) content. These fibroblasts, known as myofibroblasts, initiate wound contraction and secrete type I collagen, leading to scar formation (Hantash et al., 2008). Interestingly, normal human endometrial stromal cells (HESs) have significantly less α-SMA when compared to endometriotic stromal cells (Yuge et al., 2007). When collagen gel contractility was measured for both cell types, endometriotic cells showed a significantly higher contractility than normal HESs (Yuge et al., 2007). These results suggest there is less myofibroblastic differentiation in normal HESs, leading to reduced scar formation. Changes in fibroblast activity may alter repair mechanisms in the endometrium leading to pathology such as endometriosis and its associated adhesions and scarring. Control

of fibroblast activity could therefore lead to novel therapeutic targets for scar limitation and adhesion prevention in women with endometriosis.

1.3.5 Angiogenesis

1.3.5.1 Angiogenesis across the menstrual cycle

Angiogenesis is the process of new blood vessel formation from pre-existing vasculature and is an essential component of the human menstrual cycle. There is general consensus that there are a minimum of three stages in the cycle when angiogenesis is likely to occur (Girling and Rogers, 2005). Firstly, at menstruation when there is rapid repair of injured blood vessels. This is usually completed by day 5 of the cycle. Secondly, in the proliferative phase when there is rapid growth of the functional layer of endometrium. Thirdly, during the secretory phase when there is coiling and maturation of the spiral arterioles and growth of the subepithelial capillary plexus. However, it has been repeatedly demonstrated that levels of endothelial cell proliferation within the human endometrium do not show any consistent pattern across the menstrual cycle (Girling and Rogers, 2005). Interpretation of these results is challenging, as samples taken from women at the same stage of the menstrual cycle are extremely variable. This may be due to differences in hormone levels at the time of sampling or a variation in the region from which the biopsy was obtained. To overcome this variation, Nayak and Brenner conducted an elegant study in the ovariectomised primate model using sequential oestradiol and progesterone implants to simulate an artificial menstrual cycle (Nayak and Brenner, 2002). Using Ki67 or bromodeoxyuridine to identify proliferating endothelial cells, the authors demonstrated a 6-fold increase in proliferation during the mid-proliferative stage (day 8-10 after progesterone withdrawal). This peak was absent in the hormone-deprived animals, indicating endothelial cell proliferation at this stage in the cycle is oestradiol dependent. No significant changes in proliferation were observed at other stages of this artificial cycle.

The finding that most vascular proliferation occurs during the mid-proliferative phase in the macaque concurs with stereological analysis of human endometrium (Gambino et al., 2002). However, examination of human endometrium suggests that there is a further peak in angiogenesis or remodelling during the secretory phase,

which is not observed in the macaque. This may be due to species variation or the constant E_2 regimen used in the macaque studies, unlike a natural menstrual cycle. Alternatively it may be vessel maturation causing the vascular changes observed during the secretory phase of the cycle. Maturation involves proliferation of vascular mural cells rather than endothelial cells and would remain undetected in a system identifying only endothelial proliferation. In the mouse model, increases in mural cell proliferation and coverage were stimulated by progesterone but not E_2, in keeping with its role in the secretory phase (Girling et al., 2007).

1.3.5.2 Modes of endometrial angiogenesis

Formation of new vessels from existing vasculature occurs by at least four methods (Figure 4). Sprouting involves breakdown of the basement membrane with endothelial proliferation and migration, leading to sprouting and lumen formation from existing vessels. Intussusception results from proliferation of endothelial cells inside a vessel causing internal division and formation of two discrete capillaries. Elongation is longitudinal growth of the vessel without formation of new vascular junctions. The fourth method involves incorporation of circulating endothelial cells into endometrial vessels (Asahara et al., 1997). Gambino *et al.* (Gambino et al., 2002) used a computerized stereological program to determine if vessel elongation played a role in human endometrial angiogenesis. They analyzed full thickness endometrial sections from five phases of the menstrual cycle to determine blood vessel length density (L_v), branch point density (N_v) and mean vessel length per branch point (L_v/N_v). This allowed differentiation between determination of peaks of endothelial cell proliferation and measuring new vessel formation. This study revealed that vessel elongation was the major mechanism by which endometrial angiogenesis occurred between the early and the mid/late proliferative phases of the cycle. In the early-mid secretory phase intussusception was found to be the predominant method (Figure 3). There were no significant differences between different areas of the endometrium. These results suggest vessel elongation takes place in the proliferative phase, whereas vascular remodelling occurs during the secretory phase.

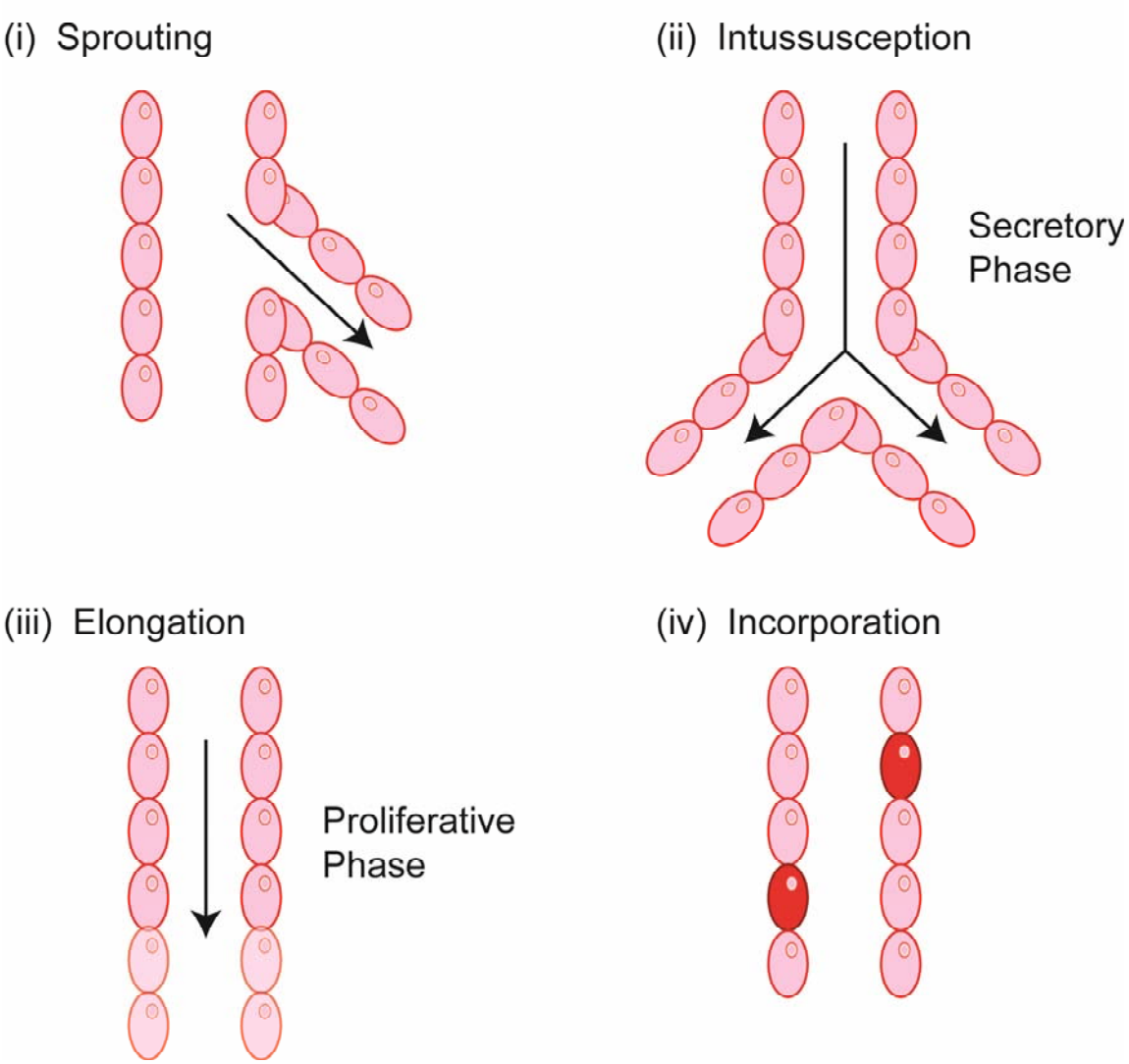


Figure 4. **Modes of angiogenesis**. (A) Sprouting involves breakdown of the basement membrane leading to lumen formation from existing vessels. (B) Intussusception results from proliferation of endothelial cells within an existing vessel to form two discrete capillaries. (C) Elongation involves longitudinal growth of a vessel. (D) Incorporation of circulating endothelial cells into an existing vessel. Adapted from (Maybin and Critchley, 2009).

1.3.5.3 Endometrial angiogenic factors

The constant angiogenic potential of the human endometrium is governed by the activity of a large number of pro- and anti-angiogenic growth factors. One of the most studied angiogenic factors is vascular endothelial growth factor (VEGF). A potent stimulator of endothelial cell proliferation, it also increases vascular permeability and has a key role in inflammation (Jabbour et al., 2006, Girling and Rogers, 2005, Ferrara, 2004, Smith, 2001). VEGF is a key factor in physiological and pathological angiogenesis (Ferrara, 2004, Carmeliet, 2005). Inactivation of a single VEGF allele results in embryonic lethality (Ferrara, 2004), indicating its fundamental role in development of the vascular system. The VEGF family is comprised of five members; VEGF-A, -B, -C, -D and placental growth factor. Of these, VEGF-A is considered the main contributor to endometrial angiogenesis and has five alternatively spliced isoforms (Girling and Rogers, 2005). VEGF-C induces endothelial cell proliferation and has been localised to uNK cells using in situ hybridisation (Li et al., 2001). VEGF-A is expressed in both the rodent and human endometrium, with localization to the stromal compartment, glandular epithelial cells and neutrophils (Ferrara, 2004, Charnock-Jones et al., 1993). VEGF-A acts via two tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), both of which are present in the human endometrium (Nayak et al., 2000, Punyadeera et al., 2006).

Early studies revealed conflicting evidence regarding changes in VEGF levels across the menstrual cycle. Some suggested an increase in protein levels during the secretory phase (Zhang et al., 1998), whereas other studies found no such difference (Li et al., 1994). Consideration of trends within different cell types and the interactions between these cells seems to have a more important influence on angiogenesis than total endometrial VEGF production. In the human endometrium, there appears to be no significant difference in stromal VEGF mRNA levels across the cycle (Gargett et al., 1999). However, neutrophils found in or around blood vessels did contain VEGF protein levels that correlated with surrounding endothelial cell proliferation (Gargett et al., 2001).

Adrenomedullin (AM) is another vasoactive peptide that belongs to the calcitonin gene peptide superfamily. It has a wide range of biological actions including vasodilation (Kitamura et al., 1993, Shindo et al., 2001, Wilkinson et al., 2001), tumour angiogenesis (Zhao et al., 1998, Oehler et al., 2002), cell growth (Nikitenko et al., 2000) and inhibition of apoptosis (Shichiri et al., 1999, Oehler et al., 2001) and is therefore a putative endometrial repair factor. AM knockout mice demonstrated embryologic lethality at approximately embryonic day 14 due to severe haemorrhage (Shindo et al., 2001). AM acts through a G protein coupled receptor, the calcitonin receptor like receptor (CLR). Receptor activity modifying proteins (RAMPs) associate with CLR to determine its ligand binding specificity (McLatchie et al., 1998). RAMP2 and RAMP3 association promotes binding of AM to CRL, while RAMP1 and CLR association promotes binding of the calcitonin gene-related peptide. AM is known to be expressed in the human endometrium (Zhao et al., 1998, Laoag-Fernandez et al., 2000) but its regulation and differential expression across the menstrual cycle has still to be fully determined.

1.4 The regulation of endometrial repair

1.4.1 Oestradiol

As E_2 is the dominant ovarian hormone in the proliferative phase, endometrial repair was traditionally thought to be an oestrogen dependent event. E_2 acts via the oestrogen receptor (ER), which has two structurally related subtypes, ERα and ERβ. Both have been described in the human endometrium (Table 1) and are expressed in the nuclei of glandular epithelial and stromal cells (Lessey et al., 1988, Critchley et al., 2002), indicating E_2 has a potential role in the regulation of repair. ERα has not been detected in endometrial vascular endothelial cells, hence E_2 was previously not considered to have a direct impact on angiogenesis. However, ERβ was subsequently detected in these cells with both polyclonal and monoclonal antibodies (Critchley et al., 2001, Lecce et al., 2001) suggesting E_2 may act directly on endometrial vessels.

To explore the role of E_2 in endometrial repair Kaitu'u-Lino *et al* utilised the murine model of simulated menstruation (Kaitu'u-Lino et al., 2007a). When progesterone support was withdrawn in ovariectomised mice, there was no significant difference in the rate of repair between mice with and without E_2 replacement. The oestrogen deplete group were maintained on a soy-free diet and injected with an aromatase inhibitor to eliminate all oestrogenic influence, demonstrating that E_2 is not essential for endometrial restoration in this model. Further evidence for oestrogen independent repair comes from observations in women following ovariectomy. Following this surgery, the endometrium has the ability to heal in the absence of ovarian hormones, providing further support for the importance of local regulation of the endometrial repair process.

Hormonal control of endometrial repair factors was further investigated in the ovariectomised macaque model (Nayak and Brenner, 2002). The authors used VEGF as an example of an endometrial angiogenic repair factor. They found three peaks of VEGF mRNA expression; in the surface epithelium during the early proliferative phase, in the stroma during the mid proliferative phase and in the glands during the late secretory phase. By comparing hormone deprived and oestrogen exposed

Table 1. Ovarian steroid receptors and prostaglandin receptors in the human endometrium across the cycle. ERα oestrogen receptor α, **ERβ** oestrogen receptor β, **PR-A** progesterone receptor A, **PR-B** progesterone receptor B, **FP** prostaglandin $F_{2\alpha}$ receptor, **EP2** a prostaglandin E_2 receptor, **LS** late secretory. (Critchley et al., 2001, Critchley et al., 2002, Lecce et al., 2001, Milne and Jabbour, 2003, Kooy et al., 1996, Jabbour et al., 2006, Milne et al., 2001, Smith et al., 2006)

Receptor	**Epithelial cell**		**Stromal cell**		**Perivascular cells**	**Vascular endothelial cells**
	Proliferative	**Secretory**	**Proliferative**	**Secretory**		
ERα	present	↓(LS)	present	↓(LS)	present	absent
ERβ	present	present	present	↓ (LS)	present	present
PR-A	present	absent	present	present	present	absent
PR-B	present	present	present	absent	present	absent
FP	present	present	absent	absent	present	present
EP2	present	present	present	present	present	present
IP	present	present	present	present	present	present

animals, the authors concluded that oestrogen is not essential for the early proliferative phase peak but is necessary for the increase in VEGF mRNA in stromal cells, seen during the mid-proliferative phase. They also reported a dramatic increase in VEGFR-1 and VEGFR-2 in the small blood vessels just below the newly formed surface epithelium during the early proliferative phase, which was independent of E_2. The E_2 dependent mid proliferative peak in VEGF expression in stromal cells correlated with a dramatic increase in endothelial cell proliferation. Of note, there was no correlation between vascular proliferation and VEGF expression in the glands or surface epithelium. The authors concluded that VEGF played a dual role in the endometrium. Firstly, a role in initial repair of damaged vessels following progesterone withdrawal in the early proliferative phase endometrium, which is oestrogen independent. Secondly, VEGF is involved in endometrial regeneration involving vascular proliferation during the mid-proliferative stage of the cycle. This latter role has an absolute requirement for oestrogen.

E_2 stimulation of VEGF expression in stromal cells has also been shown in the mouse model (Ma et al., 2001) and with *in vitro* experimentation (Huang et al., 1998). Other groups have suggested that it is the endometrial epithelial cells that are regulated by E_2 to increase VEGF expression (Albrecht et al., 2003). Human myometrial microvascular endothelial cells cultured with human recombinant VEGF were demonstrated to increase their tube formation by approximately 65% over formation in media only. This increase was replicated when the endothelial cells were cultured with endometrial epithelial cells. A further increase was seen when E_2 was added to the co-culture. No such increases in tube formation were seen when stromal cells were co-cultured with endometrial epithelial cells.

Taken together, these results suggest that E_2 has an important role in endometrial proliferation and remodelling but may not be essential for the initial repair of menstrual endometrium. Therefore, rather than increasing E_2 levels, the initiation of endometrial repair may be due to progesterone withdrawal in the late secretory phase.

1.4.2 Progesterone and its withdrawal

Progesterone is the dominant hormone of the secretory phase of the cycle. It has anti-inflammatory properties and governs differentiation of the endometrium in preparation for implantation. In the absence of pregnancy, progesterone levels fall dramatically due to pre-menstrual luteolysis and at the time of endometrial repair progesterone levels are low. There are two progesterone receptor isoforms, PRA and PRB, which are differentially regulated in the human endometrium (Table 1) (Mote et al., 2001, Mulac-Jericevic et al., 2000). During the secretory phase PRB is down-regulated in both glandular and stromal cells, whereas PRA persists in the stromal compartment (Brosens et al., 1999, Wang et al., 1998b, Mote et al., 2001).

Rodent studies have revealed progesterone has a possible stimulatory role for repair factors (Ma et al., 2001, Hyder et al., 2000). After an injection of progesterone, a small but persistent rise in VEGF occurred in the stromal cell compartment. Therefore progesterone may maintain VEGF expression throughout the menstrual cycle.

The withdrawal of progesterone in the late secretory phase, rather than its presence, is more likely to have a significant impact on endometrial repair factors. Proliferative human endometrial explants cultured *in vitro* with 17β-oestradiol and progesterone or in the absence of ovarian steroids were subjected to microarray analysis (Gaide Chevronnay et al., 2010). Functional analysis of differentially expressed genes revealed that in the absence of hormones "wound healing and inflammation" was a top scoring biological process. Studies in ovariectomised primates also demonstrate that progesterone withdrawal is a stimulus for VEGF expression (Nayak and Brenner, 2002). In addition, VEGF receptor (VEGFR-2) mRNA was dramatically up-regulated in human and macaque endometrium in stromal cells of the superficial endometrial zones during the premenstrual phase, i.e. the time of progesterone withdrawal (Nayak et al., 2000). Promatrix metalloproteinase-1 (MMP-1) was coordinately up regulated in the same stromal cell population, leading the authors to hypothesize that VEGF-VEGFR-2 interactions may influence MMPs in the upper

zones of the endometrium during the premenstrual phase, and that these interactions might play a role in induction of menstruation and/or endometrial repair.

It remains to be determined whether progesterone withdrawal increases repair factors directly, due to removal of an inhibitory influence of progesterone, or as a result of its downstream effects. Progesterone withdrawal is known to up-regulate cyclo-oxygenase-2 (COX-2), the enzyme that drives production of prostaglandin E_2 and $F_{2\alpha}$ (Hapangama et al., 2002, Critchley et al., 1999). Inhibition of COX-2 in COX-1 (-/-) null mice induced complete reproductive failure, suggesting a lack of alternative methods of prostaglandin production (Reese et al., 2001). Both PGE_2 and $PGF_{2\alpha}$ appear to be essential for normal endometrial function. Mice lacking prostaglandin receptors display significant reproductive defects. Loss of EP2, a PGE_2 receptor, resulted in impaired ovulation and reduced litter size (Tilley et al., 1999, Kennedy et al., 1999). Gene ablation of the FP receptor, the receptor for $PGF_{2\alpha}$, resulted in loss of parturition (Sugimoto et al., 1997). In addition, there is mounting evidence that the eicosanoids contribute to the processes involved in endometrial repair.

PGE_2 has been shown to promote expression of pro-angiogenic factors such as VEGF in the mouse and in human endometrial cells (Seno et al., 2002, Sonoshita et al., 2001, Sales et al., 2004). In addition, PGE_2 induced migration and tube formation of murine pulmonary microvascular endothelial cells *in vitro* and increased angiogenesis *in vivo* (Rao et al., 2007). PGE synthase and synthesis of PGE_2 have been localised to glandular epithelial and endothelial cells throughout the human endometrium and to stromal cells in the functional layer (Milne et al., 2001). PGE_2 acts via the G-protein-coupled receptor EP, of which there are four subtypes, termed EP1, EP2, EP3 and EP4. EP2 and EP4 activate adenylate cyclase and the cAMP/protein kinase A pathway, whereas EP1 and EP3 activate phospholipase C and mobilise the inositol triphosphate pathway (Figure 3)(Coleman et al., 1994). EP2 and EP4 have been detected in the human endometrium across the menstrual cycle (Milne et al., 2001), localised by *in situ* hybridization to the glandular epithelial and vascular cells.

$PGF_{2\alpha}$ is highly expressed in the human myometrium, where its well characterised function is to induce myometrial contractility (Senior et al., 1992, Sugimoto et al., 1997). In addition to its presence in the myometrium, $PGF_{2\alpha}$ is also produced from human endometrial explants cultured *in vitro* (Abel et al., 1980, Leaver and Richmond, 1984). $PGF_{2\alpha}$ is emerging as a potential key player in the initiation of endometrial repair factor expression. $PGF_{2\alpha}$ has been demonstrated to induce a host of endometrial angiogenic factors, including VEGF, FGF-2 and IL-8 (Sales et al., 2005, Sales et al., 2009, Keightley et al., 2010). Functionally, $PGF_{2\alpha}$ increased endometrial epithelial cell proliferation in culture (Milne and Jabbour, 2003) and is known to induce vasoconstriction of endometrial spiral arterioles (Baird et al., 1996). This vasoconstriction may result in a transient, local hypoxic insult in the uppermost endometrial layer. Therefore, $PGF_{2\alpha}$ may increase repair factors directly and may also indirectly up-regulate these factors as a result of induction of hypoxic conditions.

Prostacyclin (PGI) is also produced by the human endometrium (Smith et al., 1981b). This prostanoid acts via a heptahelical G protein-coupled receptor (IP receptor) (Battersby et al., 2004). Knockout studies in mice have demonstrated a role for PGI in prevention of thrombosis and increased inflammation (Murata et al., 1997, Ueno et al., 2000). Therefore, this prostanoid may be involved in menstruation and endometrial repair. Endometrial expression of the IP receptor has been demonstrated at maximal levels during menstruation (Smith et al., 2006) and was localized to epithelial, stromal and perivascular cells. Furthermore, treatment of endometrial explants with a PGI analogue significantly increased expression of proangiogenic genes (Smith et al., 2006).

1.5 The role of hypoxia in repair

1.5.1 Evidence for the presence of endometrial hypoxia

It is postulated that a period of transient hypoxia occurs in the uppermost endometrial zones following progesterone withdrawal, due to constriction of the spiral arterioles. Evidence for vasoconstriction in the perimenstrual period comes from early studies by Markee in the rhesus monkey (Markee, 1940). Endometrial explants were transplanted into the anterior chamber of the eye and vascular changes observed following progesterone withdrawal. Early changes included shrinkage of the stroma, increased spiral arteriole coiling and vascular stasis. After 24 hours, there was intense vasoconstriction, tissue necrosis and menstruation. More recently, the use of pimonidazole (hydroxyprobe®) in the mouse model of simulated menstruation identified the presence of hypoxia during menstruation (Fan et al., 2008). Pimonidazole is a marker of oxygen partial pressures less than 10mmHg and was detected in the uppermost endometrial zones during the simulated menstrual phase. In contrast, negligible levels were observed by day five post progesterone withdrawal. There is evidence that this transient hypoxic episode is also likely to occur in human endometrial tissue. Markers of tissue hypoxia (CAIX and hypoxia inducible factor-1α) have been detected immunohistochemically in the human endometrium during menstruation, with a distinct reduction in staining of both markers after cycle day 5 (Critchley et al., 2006b, Punyadeera et al., 2006).

Confirmation of human endometrial vasoconstriction *in vivo* is technically challenging. Using laser Doppler fluxmetry to measure the number of red blood cells transiting a monitored volume per unit time, Gannon *et al* failed to detect any episodes of ischaemia during menstruation (Gannon et al., 1997). This finding may be due to the limited spatial resolution of fluxmetry. Focal or prolonged ischaemia-reperfusion episodes would not be detectable with this method, particularly if episodes last longer than the authors' 10min sampling time. In addition, the study does not state exclusion of women with HMB. There is some evidence that women with HMB have reduced levels of endometrial vasoconstrictors in the perimenstrual phase, such as endothelin-1 (Marsh et al., 1997, Smith et al., 1981a). In addition,

women with HMB have been shown to have increased uterine blood flow, quantified using pulsatility index measurement (Hurskainen et al., 1999). Therefore aberrant vasoconstriction in these women may skew results, preventing the accurate delineation of normal endometrial physiology and detection of a reduction in endometrial perfusion during menses.

Early studies detecting vasoconstriction of spiral arterioles resulted in the hypothesis that endometrial hypoxia stimulates menstruation (Markee, 1940). As MMPs are responsible for the degradation of endometrial tissue at menstruation, it was subsequently proposed that hypoxia up-regulated MMP expression. However, recent evidence raises questions about this theory. Primary human endometrial stromal cells cultured in normoxic (21% O_2) and hypoxic (2% O_2) conditions for 24 and 48 hours revealed that hypoxia in fact decreased the secretion of membrane-type 1 MMP, active MMP-2, proMMP-1 and proMMP-3 (Zhang and Salamonsen, 2002). Similar decreases in MMPs were also observed in the culture supernatants from whole endometrial explants cultured in 0.1% O_2 for 24 hours (Gaide Chevronnay et al., 2010). These results suggest that hypoxia is not responsible for endometrial breakdown and support the "tissue inflammation and destruction" hypothesis for the initiation of menses. However, hypoxic conditions may have a fundamental role in the initiation of endometrial repair.

1.5.2 Hypoxia inducible factor

Hypoxia-inducible factor (HIF) is a transcription factor that controls the cellular adaptive response to hypoxia. It is composed of two subunits; the oxygen-destructible α-subunit and the constitutively expressed β-subunit. Three isoforms of the α-subunit (HIF-1α, HIF-2α, and HIF-3α) have the ability to bind to HIF-1β to modulate the *in vivo* response to hypoxia. HIF-1α-HIF-1β and HIF-2α-HIF-1β heterodimers have overlapping but distinct target gene specificities (Ratcliffe, 2007, Sowter et al., 2003). In contrast to HIF-1α, HIF-2α is not expressed in every cell type. HIF-3α is much less studied but appears to have an inhibitory action on HIF activity (Makino et al., 2001, Makino et al., 2007). HIF-1α and HIF-1β contain basic helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS) domains that mediate heterodimerization and DNA binding (Figure 5). HIF-1α also contains two

transactivation domains (TAD) that interact with co-activators such as p300 and CREB binding protein (CBP) (Kallio et al., 1998, Jiang et al., 1997). When αβ dimerisation occurs, HIF translocates to the nucleus and initiates the transcription of genes with hypoxic response elements (Figure 6). Target genes of HIF include those involved in tissue remodelling, apoptosis, energy metabolism and angiogenesis (e.g. VEGF, CTGF, Ang-2, GAPDH, AM, CXCL8) (Semenza, 2000, Higgins et al., 2004, Cormier-Regard et al., 1998, Graven et al., 1999).

1.5.2.1 Regulation of HIF by oxygen

The activity of the oxygen destructible alpha subunit of HIF is regulated by post-translational mechanisms (Figure 6). In the presence of oxygen, two prolyl residues are hydroxylated in its oxygen-dependent degradation domain. This targets HIF-1α for proteasomal degradation by promoting ubiquitylation and interaction with the von Hippel-Lindau protein (Jaakkola et al., 2001, Ivan et al., 2001). In addition, hydroxylation of an asparagine residue in the c-teminus of the alpha subunit inhibits the binding of coactivators (p300/CBP) (Dann et al., 2002). Oxygen is required as a co-substrate for the enzymes carrying out these hydroxylation reactions, the prolyl hydroxylases (PHD). The reaction also requires an Fe(II) ion cofactor and 2-oxoglutarate but oxygen appears to be the rate limiting factor for enzymatic activity in physiological conditions (Semenza, 2004). In hypoxic conditions hydroxylation cannot take place, resulting in rapid HIF-1α accumulation. On re-oxygenation HIF-1α is degraded with a half-life of less than 5min (Wang et al., 1995).

1.5.2.2 Non hypoxic activation of HIF

In addition to activation by hypoxia, many inflammatory and growth factors have been shown to influence HIF-1 activity, including TNFα, IL-1β, PGE_2 and NFκB (Hellwig-Burgel et al., 1999, Critchley et al., 2006b, Haddad and Land, 2001, van Uden et al., 2008). Unlike hypoxic conditions, the main mechanism by which these factors induce HIF-1 activity is by increasing HIF-1α protein translation, rather than protein stabilisation (Frede et al., 2005, Page et al., 2002). An increase in HIF-1α mRNA transcription has also been demonstrated in the non-hypoxic induction of HIF-1α (Blouin et al., 2004, Rius et al., 2008). HIF-1α protein degradation appears to be relatively unaffected by these factors. Increases in transcription and translation of

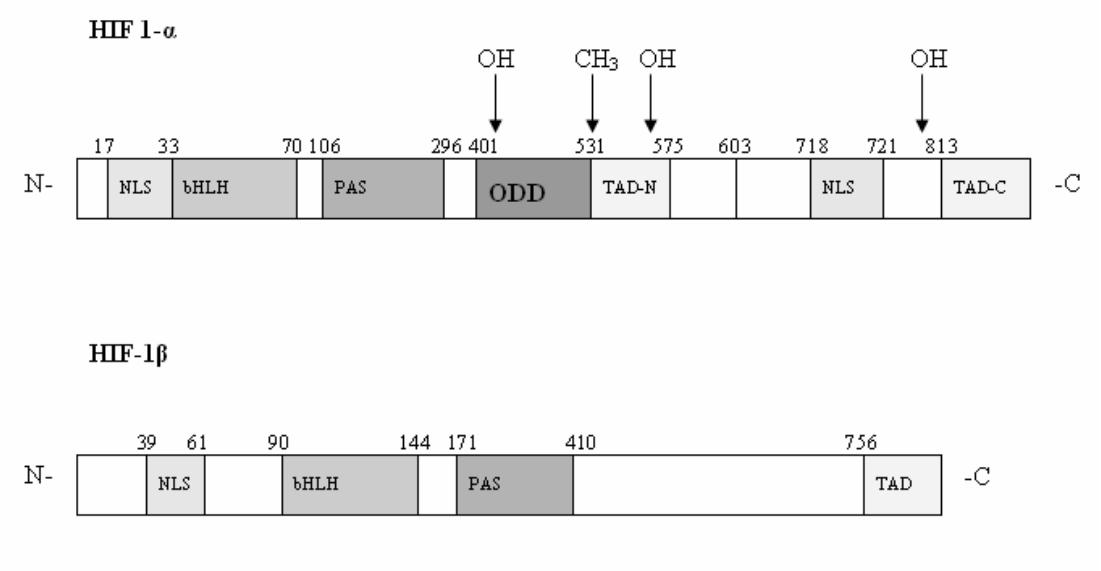


Figure 5. **HIF-1α and HIF-1β protein structure.** Both proteins contain transactivation domains (TAD) which regulate the transcription of HIF-1 target genes and also bind co-activators such as p300/CBP and Ref-1 to increase HIF transcription. Both HIF-1α and HIF-1β contain a basic helix-loop-helix (bHLH) domain essential for DNA binding. The bHLH domain is also responsible for subunit dimerisation. HIF-1α contains an oxygen dependent degradation domain (ODD) through which the degradation of HIF-1α in normoxia is triggered. (PAS Per-ARNT-Sim domain, NLS nuclear localisation signal).

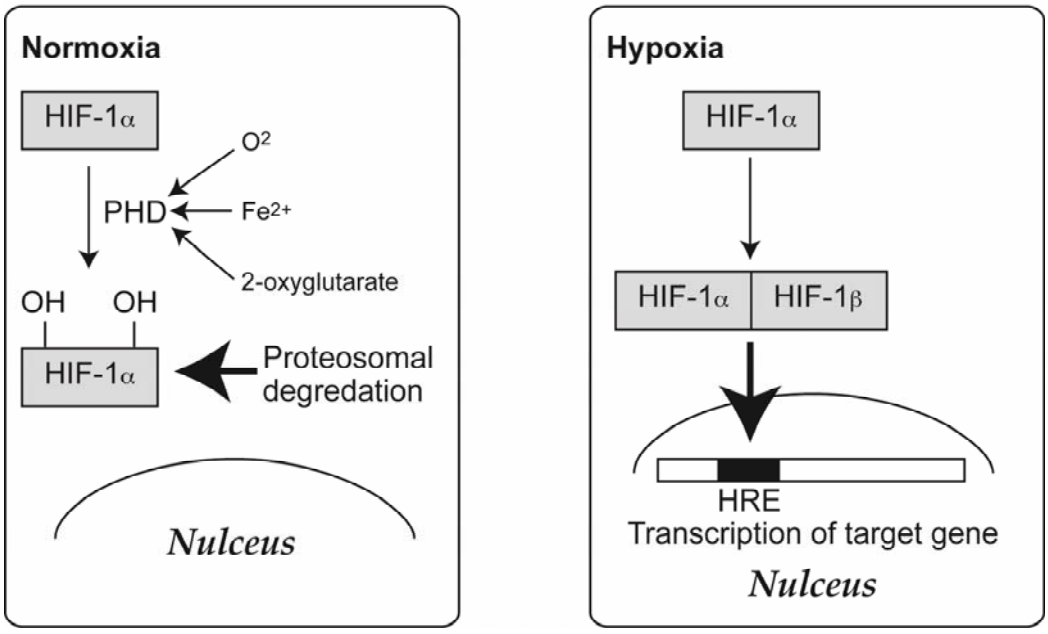


Figure 6. **Hypoxia inducible factor -1 (HIF-1) activity in normoxic and hypoxic conditions.** In normoxia, hydroxylation of HIF-1α at two proline residues promotes its proteosomal degradation. Hydroxylation is mediated by prolyl hydroxylases (PDH), which require an iron ion cofactor (Fe^{2+}), 2-oxoglutarate and oxygen. In hypoxic conditions these processes are suppressed, allowing HIF-1α to dimerise with HIF-1β, translocate to the nucleus and bind to hypoxic response elements (HRE) on target genes to induce transcription.

HIF-1α mRNA appears sufficient to increase HIF-1α protein levels by shifting the balance towards HIF-1α synthesis to surpass its degradation.

1.5.2.3 HIF in the human endometrium

HIF-1α has been identified in the human endometrium and has an attractive role in the co-ordination of angiogenic and mitogenic factors for endometrial repair. HIF-1α was demonstrated by immunohistochemistry to be present exclusively in the late secretory and menstrual phases (Critchley et al., 2006b). It was localised to the surface epithelial, glandular and stromal cells in the functional layer, with staining limited to the glandular cells in the basal layer. Interestingly, HIF-1α protein co-localised with the EP2 receptor, suggesting up-regulation by PGE_2. This was confirmed by HIF-1α protein detection in endometrial cells treated with 100nM PGE_2 in normoxic conditions but not those treated with vehicle. This PGE_2 induction of HIF-1α in normoxic conditions has been previously demonstrated in prostate cancer cells and colon cancer cells (Liu et al., 2002, Fukuda et al., 2003). In addition, HIF-1α has been shown to regulate the endometrial angiogenic factor cysteine rich protein 61 (CYR61) (Gashaw et al., 2008). This pro-angiogenic factor was up-regulated at the mRNA and protein level during menstruation. Hypoxic conditions increased endometrial cell CYR61 mRNA expression and secreted protein levels. Silencing of HIF-1α in these cells using short interfering RNA (SiRNA) resulted in a significant reduction in hypoxia-induced CYR61 mRNA and protein when compared to cells transfected with control SiRNA.

Cognisant of the contribution of HIF to the up-regulation of factors involved in metabolism, angiogenesis and extracellular matrix remodelling in other systems (Semenza, 2004), it has an attractive role as the master regulator of endometrial repair. Its endometrial induction may be classical, due to a transient hypoxic episode, or due to the inflammatory mediators present during menstruation. The contribution and presence of HIF-2α in the endometrium is similarly unknown and endometrial target genes of the HIF heterodimer are still to be elucidated. Not only has HIF-1 a potential role in the repair and remodelling of the normal human endometrium but its aberrant expression may contribute to defective repair and the common gynaecological complaint of heavy menstrual bleeding.

1.6 Heavy menstrual bleeding

1.6.1 Definition of heavy menstrual bleeding (HMB)

HMB is defined as excessive menstrual blood loss that interferes with a woman's physical, emotional, social and/or material quality of life, (NICE, 2007). This subjective definition of HMB is useful clinically as it is the woman's perception and ability to cope with her bleeding that determines the need for treatment, rather than the actual volume of menstrual blood loss (MBL) (Warner et al., 2004). In contrast, high individual variability in the subjective definition of HMB poses problems for clinical research into this disorder. Therefore, HMB can also be defined objectively as a monthly menstrual blood loss (MBL) of greater than 80ml (Hallberg et al., 1966a). Menstrual fluid is composed of blood, endometrium and a serous endometrial transudate, but objective measurements are usually based on menstrual haemoglobin content alone. Early Scandinavian studies demonstrated that the mean MBL was 40ml and that regular loss greater than 63ml was associated with iron deficiency anaemia (Hallberg et al., 1966a, Hallberg et al., 1966b, Hallberg and Nilsson, 1964). The 90^{th} centile for MBL in these studies was 80ml, and this has been adopted as the lower limit at which blood loss is traditionally defined as heavy.

1.6.2 The significance of HMB

HMB is a common condition in women of reproductive age. One in three women finds their menstrual loss excessive, with this figure rising to one in two as the menopause approaches (Prentice, 1999). Over 800 000 women seek treatment per year in the UK alone (NICE, 2007) and menstrual complaints constitute greater than 20% of all secondary referrals to gynaecology outpatient clinics (Santer et al., 2005). Considering almost 70% of women between 16 and 59 in the UK are in employment (2000), this debilitating condition has a significant socio-economic impact. Assessment of the economic impact of menstrual complaints in the US demonstrated financial losses of greater than $2000 per patient per year due to absence from work and home management costs (Frick et al., 2009). Although medical treatment options are available for HMB, many women proceed to surgery due to medical treatment failure or side effects. Hysterectomy remains one of the most commonly performed

surgical procedures (NICE, 2007), with HMB a leading indication. The English rate of surgery for heavy menstrual bleeding is 140 women per 100 000 (Cromwell et al., 2009). Annual treatment costs exceed £65 million and women are subjected to all the risks associated with surgical procedures (Weeks et al., 2000). It is essential to fully understand the pathogenesis of HMB to improve medical treatments and avoid the risks of surgery. Ideally treatments for HMB would be non-hormonal, to avoid troublesome side effects and exclusion of their use in women wishing to conceive.

1.6.3 Pathogenesis of HMB

A new classification system for abnormal uterine bleeding has recently been outlined, describing bleeding due to polyps, adenomyosis, leiomyoma, malignancy, coagulopathy, ovulatory dysfunction, endometrial, iatrogenic and not-yet classified (the PALM-COEIN system) (Munro et al., 2011). However, approximately 50% of cases of HMB occur in the absence of recognised pathology. In such circumstances HMB is likely to be a consequence of disturbance of local endometrial mechanisms leading to increased flow and/or prolonged bleeding. Observational studies have reported the typical normal duration of bleeding to be from three to eight days (Treloar et al., 1967, Harlow and Campbell, 1994). Prolonged menstrual bleeding (>eight days) may be due to excessive inflammation at menstruation, defective endometrial homeostatic mechanisms or delayed repair of endometrial vessels and tissue. Increased flow at menstruation is likely to be due to impaired vasoconstriction of the specialised endometrial spiral arterioles.

1.6.3.1 Impaired vasoconstriction

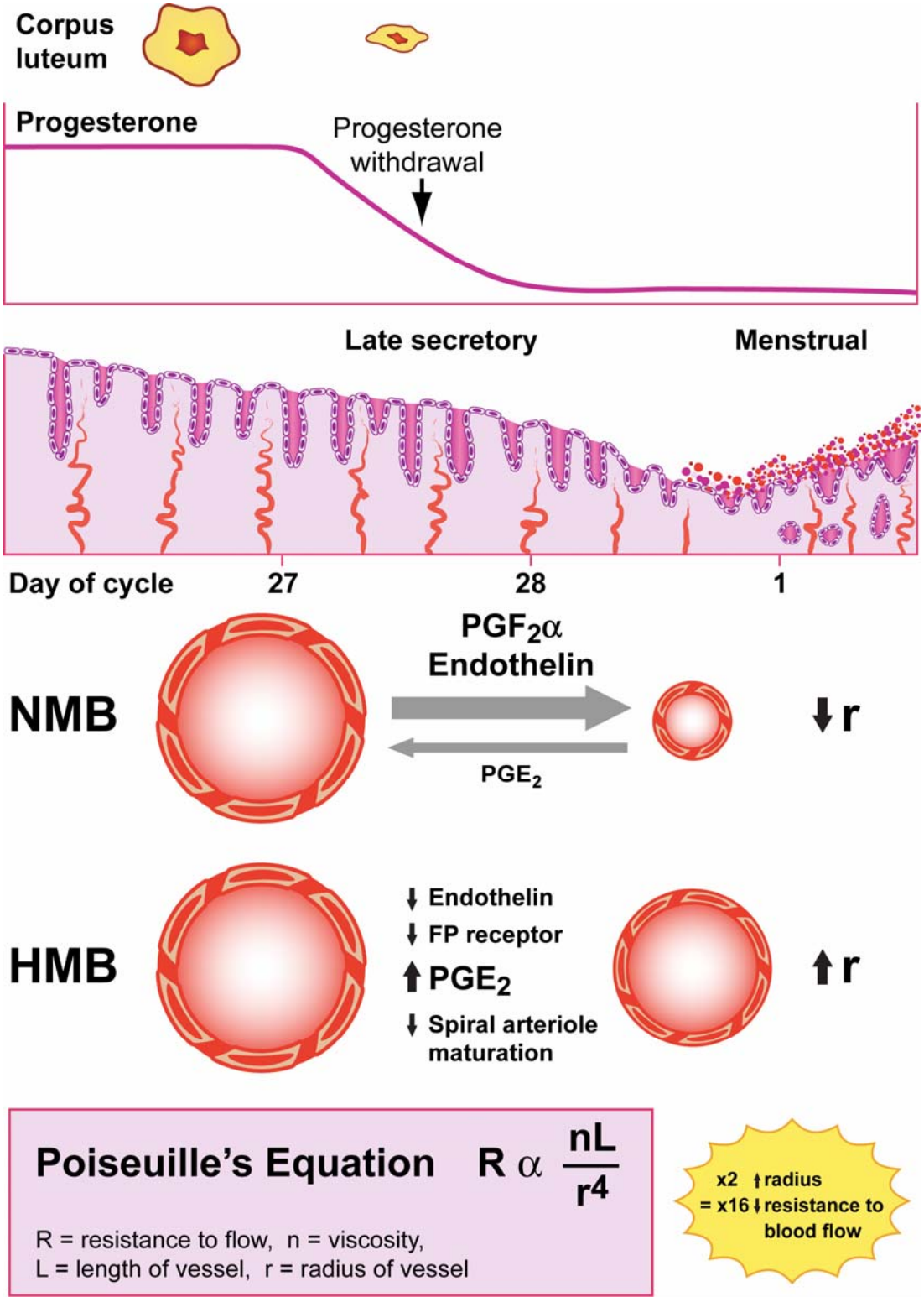
As the radius of a vessel is the major determinant of resistance to flow, decreased constriction of endometrial vessels at the time of menstruation will contribute significantly to increased blood loss (Figure 7). $PGF_{2\alpha}$ and endothelin-1 (ET-1) are two endometrial factors with known vasoconstrictive properties (Baird et al., 1996, Marsh et al., 1997). Women with menstrual blood loss in excess of 90ml have been shown to have a significantly decreased $PGF_{2\alpha}/PGE_2$ ratio (Smith et al., 1981a) and decreased FP receptor expression (Smith et al., 2007). Excessive PGE_2 production at the expense of $PGF_{2\alpha}$ may result in less constriction of the spiral arterioles prior to menstruation. In addition, women with HMB have decreased endometrial expression

of the potent vasoconstrictor ET-1 and increased levels of its metabolising enzyme, neural endopeptidase (Marsh et al., 1997). The authors suggest that increased metabolism of endothelin could explain its decreased endometrial expression and cause dilation of endometrial vessels at menstruation. Altered spiral arteriole maturation may also contribute to inefficient spiral arteriole vasoconstriction at menstruation. Vessel wall circumference and focal discontinuities were noted to be larger in the endometrium of women with HMB than normal controls (Mints et al., 2007). Women with heavy bleeding had significantly reduced vascular smooth muscle cell proliferation in spiral arterioles during the mid-late secretory phase when compared to normal controls (Abberton et al., 1999b). In addition, smooth muscle myosin heavy chain, a contractile protein used as a marker of vascular smooth muscle cell maturation, was also significantly decreased in spiral arterioles of women with HMB (Abberton et al., 1999a). Taken together, the decreased levels of vasoconstrictive factors and immature vessels present in women with HMB will significantly increase MBL. Application of Poiseuille's equation (Figure 7) reveals that a two fold increase in vessel radius leads to a sixteen fold decrease in resistance to flow, highlighting that even small changes in endometrial vasoconstriction will lead to a dramatic increase in menstrual loss.

1.6.3.2 Defective homeostasis

In other tissues, disruption of blood vessels after injury results in adherence of platelets to collagen on the injured basement membrane. Platelet aggregation stimulates the coagulation cascade and formation of a fibrin clot. In contrast, studies of the endometrium suggest that platelet involvement is relatively low and that vasoconstriction and the clotting cascade are more important in achieving homeostasis post-menstruation (Gelety and Chaudhuri, 1995). The coagulation cascade is activated by two pathways; extrinsic and intrinsic (Figure 8). Each culminates in the conversion of factor X to Xa, which catalyses the conversion of pro-thrombin to thrombin, ultimately leading to the formation of a fibrin clot. Disorders that interfere with systemic haemostasis have an impact on MBL. Von Willebrand disease is the most common of these disorders, with a prevalence of 13% in women with a complaint of HMB (Shankar et al., 2004).

Figure 7. **The influence of vessel radius on blood flow at menstruation**. Progesterone levels decrease during the late secretory phase as the corpus luteum regresses. In women with normal menstrual bleeding (NMB), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and endothelin-1 cause vasoconstriction of the spiral arterioles at menstruation to decrease blood flow. Women with heavy menstrual bleeding (HMB) have been shown to have lower levels of FP receptor (Smith et al., 2007), endothelin-1 (Marsh et al., 1997), and spiral arteriole maturation (Abberton et al., 1999a). In addition, they have higher levels of the vasodilator, prostaglandin E_2 (Smith et al., 1981a). The resulting lack of vasoconstriction has a profound effect on blood loss, illustrated by Poiseuille's equation (Maybin et al., 2010).


Corpus luteum
Progesterone
Progesterone withdrawal
Late secretory
Menstrual
Day of cycle
27
28
1
NMB
PGF2α
Endothelin
PGE2
↓r
HMB
↓ Endothelin
↓ FP receptor
↑ PGE2
↓ Spiral arteriole maturation
↑r
Poiseuille's Equation
R α nL / r4
R = resistance to flow, n = viscosity,
L = length of vessel, r = radius of vessel
x2 ↑radius
= x16 ↓resistance to blood flow

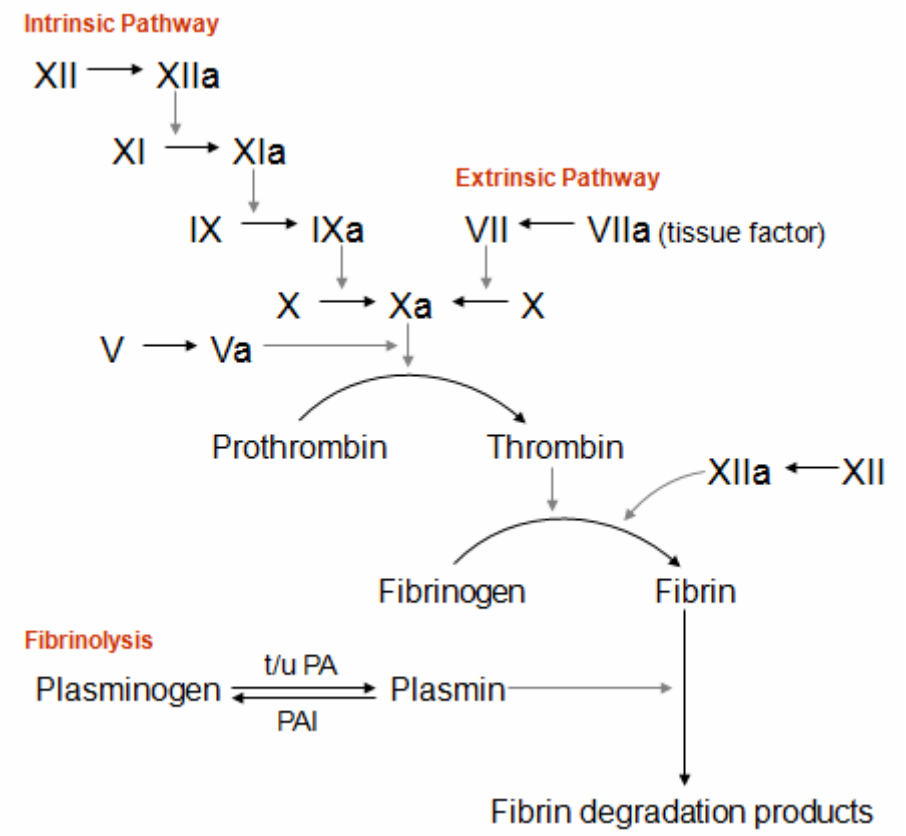


Figure 8. **Haemostasis: the coagulation cascade and fibrinolytic system.** Activation of coagulation occurs by two pathways, extrinsic and intrinsic. Conversion of fibrinogen to fibrin allows formation of a haemostatic clot. Breakdown of this clot is mediated by the fibrinolytic system. Some women with heavy menstrual bleeding have been shown to have increased endometrial levels of tissue plasminogen activator (tPA) (Gleeson et al., 1993), leading to high plasmin levels and increased breakdown of the haemostatic clot.

Fibrin deposits are essential for homeostasis but inadequate resolution of the deposit can result in scarring. Degradation of the fibrin clot is mediated by the fibrinolytic system (Figure 8). After peritoneal surgery there is defective endogenous fibrinolysis, which may explain the high level of adhesion formation observed post-operatively (Hellebrekers et al., 2000). Fibrinolysis involves conversion of plasminogen to active plasmin, promoting the degradation of fibrin deposits. Tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) drive the production of plasmin. In contrast, plasminogen activator inhibitor (PAI) inhibits fibrinolytic activity. The human endometrium contains t-PA and u-PA, as well as PAI and the uPA receptor (Gleeson et al., 1993, Nordengren et al., 2004). An active fibrinolytic system at menstruation may confer protection against scarring during endometrial repair. However, an overactive fibrinolytic system may interfere with haemostasis and contribute to HMB. Women suffering from heavy menstrual loss have raised levels of t-PA activity on the second day of bleeding when compared to those with normal loss (Gleeson et al., 1993). Further evidence for this over activation of the fibrinolytic system comes from the efficacy of tranexamic acid as a treatment for HMB. This antifibrinolytic reduces t-PA and PAI levels in women with HMB and results in a 58% reduction in blood loss (Gleeson et al., 1994). Of note, women taking tranexamic acid do not suffer from scarring or adhesion formation within the endometrium, suggesting that the balance between PA and PAI may be more important in scar prevention than absolute levels.

1.6.3.3 Excessive menstrual inflammation

The finding of increased levels of the pro-inflammatory cytokine TNFα in the menstrual effluent of women with HMB compared to normal controls lends support to the hypothesis that excessive inflammation can contribute to HMB (Malik et al., 2006). The role of the prostaglandin synthesis pathway has also been examined in women with objective measurement of their menstrual blood loss. Analysis of gene expression in endometrial biopsies from the secretory phase revealed significant elevation of COX-2 mRNA expression in women with blood loss greater than 80ml (Smith et al., 2007). Increased levels of total prostaglandins have been found in the endometrium of women with HMB (Smith et al., 1981a, Smith et al., 1981b). Increased signalling of PGE_2 through its EP2 and EP4 receptors has also been

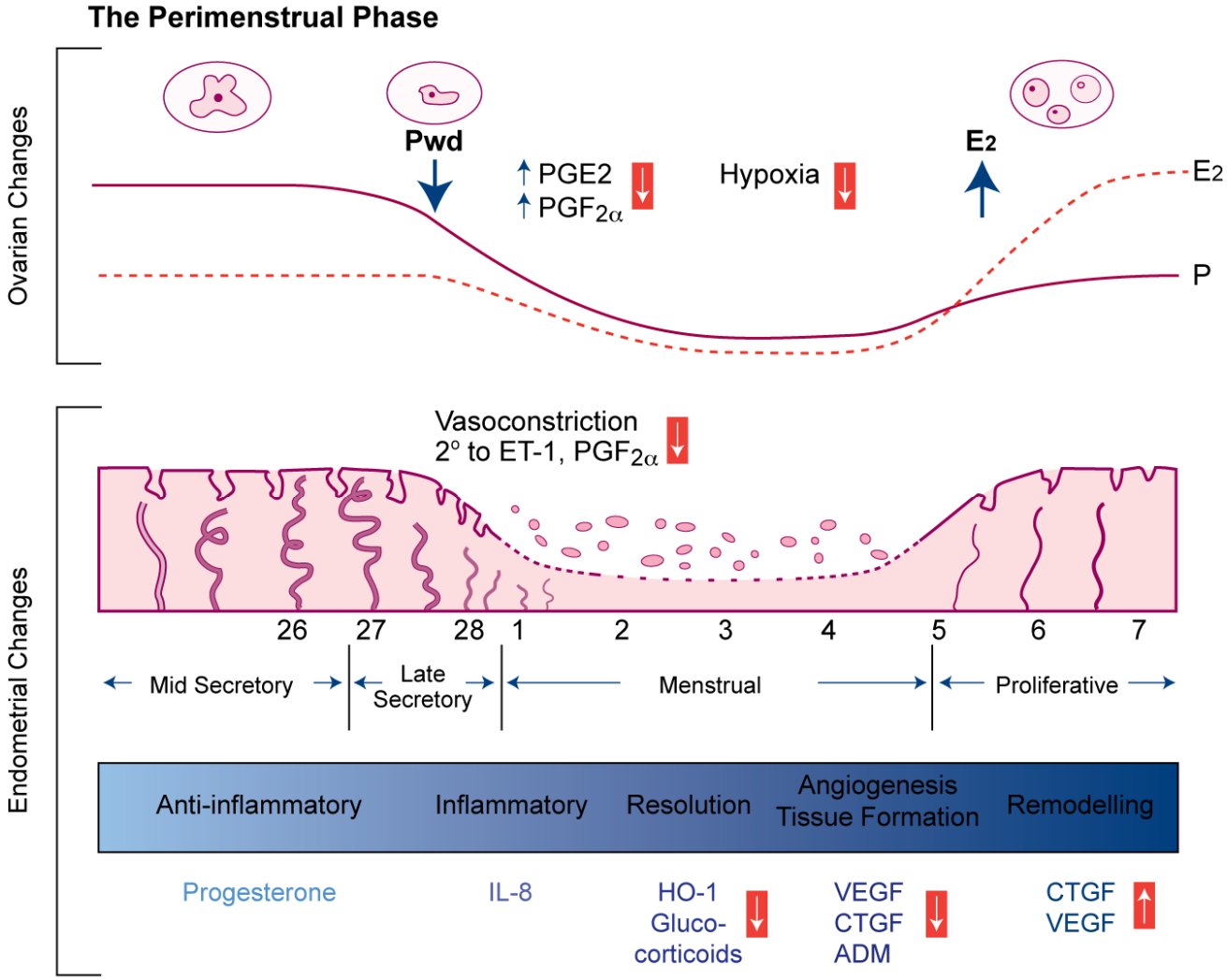
suggested due to elevated production of cyclic AMP in endometrium from women with heavy versus normal bleeding. Furthermore, the enzyme involved in cAMP hydrolysis, phosphodiesterase E4, was decreased in women with HMB (Smith et al., 2007). These findings suggest excessive prostaglandin production and signalling in the endometrium of women with HMB. The resulting exaggerated inflammation may lead to increased and prolonged tissue damage at the time of menstruation. Currently prostaglandin synthesis inhibitors are a useful medical treatment for reduction of menstrual blood loss (Cameron et al., 1990). However, although women treated with mefenamic acid had a significant decrease in menstrual loss, 52% maintained a blood loss greater than 80ml after two months of treatment (Cameron et al., 1990).

1.6.3.4 Delayed endometrial repair

Lack of vasoconstriction during menstruation may not only increase menstrual flow but also limit or prevent the perimenstrual hypoxic insult in the upper endometrial zones of women with HMB. This may impact on the initiation of endometrial repair. Decreased activation of HIF-1 may prolong menstrual bleeding if there is a subsequent decrease in the transcription of angiogenic repair factors. VEGF is an angiogenic factor that is a well established target gene of HIF-1 (Fukuda et al., 2003). Women with blood losses in excess of 80ml have been shown to have significantly lower VEGF mRNA at menstruation than women with a loss less than 80ml (Malik et al., 2006). VEGF promotes the migration and differentiation of vascular smooth muscle cells (Arkonac et al., 1998) and contributes to initiation of coagulation (Blum et al., 2001). Decreased VEGF expression at menstruation may delay repair of damaged vessels and increase menstrual loss.

Evidence for the need for controlled perimenstrual inflammation, followed by well timed resolution and repair is mounting. Unrestrained inflammation or aberrant repair may both contribute to the onset of HMB (Figure 9). Treatment of women with resistant HMB may require maximisation of the repair process in addition to current treatments that focus on limitation of the inflammatory response. Further research into the pathways involved in endometrial repair, including the contribution of HIF-1 and NFκB, may identify potential targets for novel therapeutic intervention in these women.

Figure 9. **Local endometrial inflammatory and repair factor expression during luteo-follicular transition** (adapted from Critchely & Maybin. Semin Reprod Med. 2011)**.** As the corpus luteum regresses during the late secretory phase, progesterone levels fall. This triggers a cascade of inflammatory mediators in the pre- and early-menstrual endometrium, including cyclo-oxygenase-2 (COX-2), interleukin-8 (IL-8) and prostaglandins (PG) E_2 and $F_{2\alpha}$. Glucocorticoids and heme-oxygenase (HO-1) may be involved in limitation of this inflammatory response. Vasoconstriction of the endometrial spiral arterioles occurs following P-withdrawal, due to increasing levels of endothelin-1 (ET-1) and $PGF_{2\alpha}$. This is thought to lead to a transient hypoxic episode in the uppermost endometrial zones, which may stimulate endometrial repair factor production. Vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), adrenomedullin (AM), and fibronectin are factors with a putative role in endometrial repair and remodelling post menstruation. There is evidence that local endometrial factors are aberrant in women with HMB, red arrows (Smith et al., 1981a, Smith et al., 2007, Marsh et al., 1997, Rae et al., 2009, Malik et al., 2006).


The Perimenstrual Phase
Ovarian Changes
Pwd
PGE2
PGF2α
Hypoxia
E2
E2
P
Endometrial Changes
Vasoconstriction
2° to ET-1, PGF2α
26
27
28
1
2
3
4
5
6
7
Mid Secretory
Late Secretory
Menstrual
Proliferative
Anti-inflammatory
Inflammatory
Resolution
Angiogenesis Tissue Formation
Remodelling
Progesterone
IL-8
HO-1
Gluco-corticoids
VEGF
CTGF
ADM
CTGF
VEGF

1.7 Hypotheses

The mechanisms involved in endometrial repair are still to be fully characterised. Likewise, the regulation of this tightly controlled, efficient process remains undefined. Delineation of the physiology of endometrial repair may lead to novel treatment strategies for the common gynaecological complaint of HMB.

1. As endometrial repair is thought to commence on day two of the cycle, during active bleeding, factors involved in this process would be expected to be increased during the menstrual phase. It is clear that inflammation, its resolution, tissue formation/remodelling, vasoconstriction and angiogenesis are all key events in the repair process. Factors with putative roles in endometrial repair include IL-8 (inflammation/angiogenesis), AM and VEGF (angiogenesis), CTGF (tissue formation/remodelling) and ET-1 (vasoconstriction).

Hypothesis: Factors involved in endometrial repair (IL-8, VEGF, AM, CTGF, ET-1) are significantly up-regulated during menstruation.

2. The predominant hormonal change in the pre-menstrual endometrium is the withdrawal of progesterone as the corpus luteum regresses. The subsequent increased synthesis of prostaglandins is well established (Baird et al., 1996). There is also evidence for a transient, local hypoxic episode in the upper endometrial zones (Critchley et al., 2006b, Fan et al., 2008). Any menstrual up-regulation of factors involved in endometrial repair is, therefore, potentially regulated by progesterone withdrawal, prostaglandins and/or hypoxia.

Hypothesis: Progesterone withdrawal, Hypoxia, PGE_2 and/or $PGF_{2\alpha}$ up-regulate endometrial repair factors.

3. The transcription factor HIF is known to regulate the cellular response to hypoxia (Maxwell, 2005). NFκB is a transcription factor with a key role in the inflammatory response (Baldwin, 1996). Both factors have been identified in the human endometrium during the late-secretory/menstrual phase (Critchley et al., 2006b, King et al., 2001). Hence, prostaglandin and hypoxia induced up-regulation of endometrial repair genes may be due to increased activity of HIF and/or NFκB.

Hypothesis: Prostaglandins and Hypoxia increase repair factor expression in the human endometrial cells via the initiation of transcription by HIF and/or NFκB.

4. Defective endometrial repair may contribute to the common gynaecological complaint of HMB. Decreased vasoconstriction, excessive inflammation or delayed vascular repair can lead to heavy or prolonged bleeding. Examination of endometrium from women with objectively measured MBL may identify aberrant gene expression and potential therapeutic targets.

Hypothesis: menstrual endometrium from women with HMB exhibits altered repair factor expression when compared to that from women with NMB.

1.8 Aims

The specific aims of this PhD were to:

1. determine the expression of putative repair factors in human endometrial biopsies from across the menstrual cycle.

2. elucidate the role of hypoxia, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ in the regulation of endometrial repair factors.

3. assess the contribution of HIF and NFκB to the regulation of endometrial repair factors.

4. examine differences in endometrial repair factor expression between women with objectively measured HMB and NMB.

2. General Materials & Methods

2.1 Patient recruitment and endometrial tissue collection

Written informed consent was obtained from 187 healthy women of reproductive age (Figure 10) (Appendix 1). Institutional ethical approval was obtained from the Lothian Research Ethics Committee (LREC/07/S1103/29) (Appendix 2). Participants were aged 22 to 50 years (median age 41; mean age 41). All reported regular menstrual cycles (cycle length 21 to 35 days) and had not taken any exogenous hormones or used an intrauterine device during the 3 months prior to tissue collection. Women with known uterine pathology such as large fibroids (>3cm) and endometriosis were excluded.

Human endometrial biopsies were collected from 179 of the 187 women at the time of hysterectomy or investigation in the gynaecology out-patient setting for a subjective complaint of HMB (Figure 10, patient recruitment flow chart). Endometrial tissue was not obtained from eight women due to technical difficulties (n=3) or failure to attend for their appointment (n=5). A further 21 tissues were excluded from the study due to inconsistent dating (n=13) or due to the subsequent finding of uterine pathology (n=8).

Endometrial biopsies were collected with an endometrial suction curette (Pipelle, Laboratorie CCD, Paris, France). Immediately after collection tissue was divided and placed in:

(i) **RNA later**, an RNA stabilisation solution (Ambion (Europe) Ltd., Warrington, UK) and stored at -70ºC for RNA extraction
(ii) **neutral buffered formalin** (NBF) to fix tissue prior to wax embedding
(iii) **phosphate buffered saline** (PBS) for *in vitro* culture.

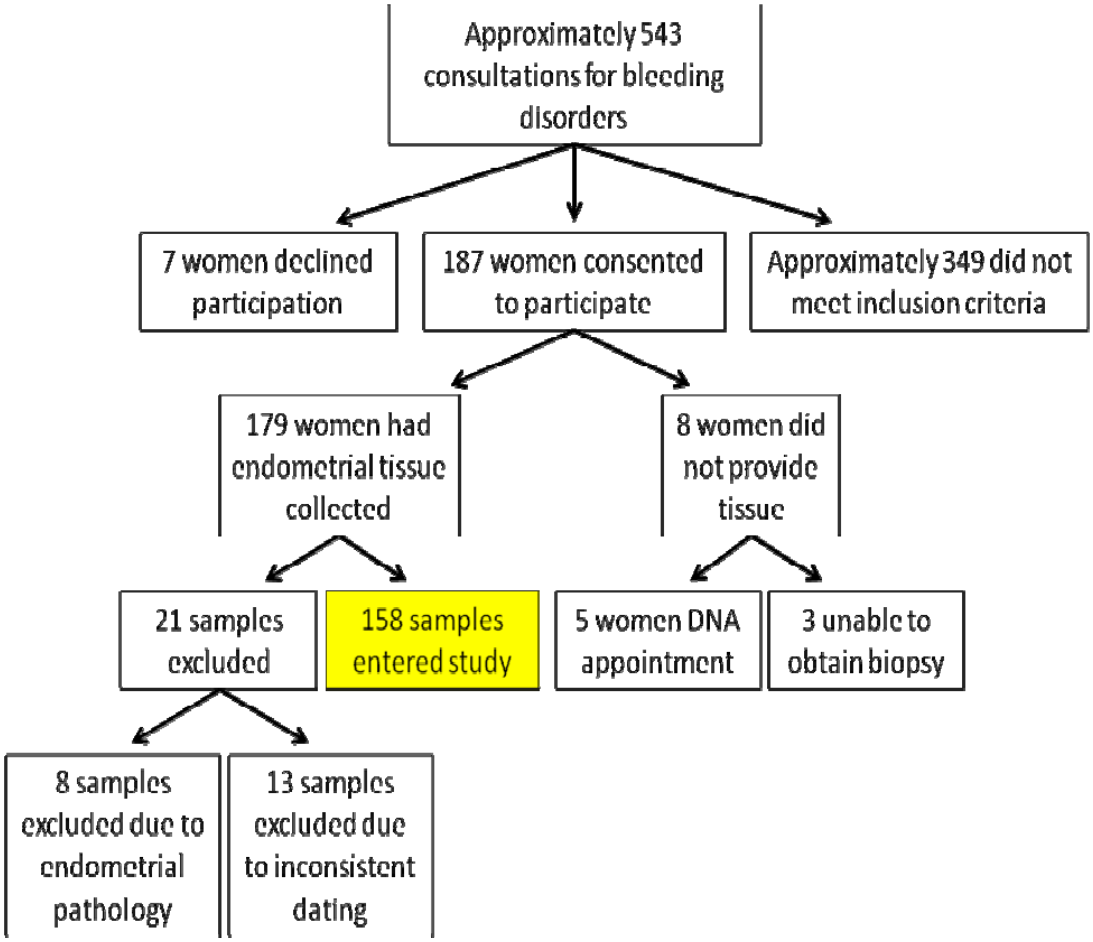


Figure 10. **Recruitment figures**, including details of exclusions. DNA: did not attend.

At hysterectomy, an additional full thickness section including the functional and basal endometrial layers and adjacent myometrium was taken from the uterine cavity and fixed in NBF for immunohistochemical studies. The biopsies were dated according to three criteria:

(i) the **histological appearance** based on the criteria of Noyes *et al*. (Noyes et al., 1950), assessed by a consultant pathologist (Dr Alistair Williams)

(ii) the participant's reported **last menstrual period** (LMP)

(iii) the circulating **serum progesterone and oestradiol levels** in serum samples collected from each woman at the time of endometrial biopsy (radioimmunoassay by Mr Ian Swanston).

Consistency for all three parameters was necessary before inclusion in the study. Biopsies were classified as proliferative (P), early secretory (ES), mid secretory (MS), late secretory (LS) or menstrual (M) for analysis (Table 2).

Table 2. Classification and clinical details of endometrial tissue samples examined in this thesis. *oestradiol values significantly decreased when compared to proliferative, early and mid-secretory values (M $p<0.001$, LS $p<0.05$). $^{\delta}$progesterone values significantly decreased compared to early and mid-secretory values (M $p<0.001$, P $p<0.01$, LS $p<0.05$).

Stage of cycle	Cycle day	No. of samples	Median Oestradiol (pmol/litre)	Median Progesterone (nmol/litre)	Median age in years (range)	Median parity (range)
Menstrual	1 to 7	18	145.2* (55-514)	2.8$^{\delta}$ (1.3-18.4)	40 (33-49)	2 (1-5)
Proliferative	5 to 18	51	498.0 (103.6-2004)	3.0$^{\delta}$ (1.0-40.8)	42 (26-50)	2 (0-5)
Early Secretory	14 to 20	28	573.1 (282.2-919.3)	41.1 (16.1-119.3)	42 (33-49)	2 (0-5)
Mid Secretory	19 to 23	42	435.0 (153.8-1949)	49.5 (8.5-262.1)	42.5 (22-52)	2 (0-3)
Late Secretory	24 to 33	19	225.5* (59.1-819)	9.7$^{\delta}$ (1.1-26.5)	41.5 (36-48)	2 (0-3)

Of the 179 women who provided tissue samples, 21 consented to return for a repeat endometrial biopsy 3-6 months following insertion of a levonorgestrel-releasing intrauterine system (LNG-IUS) for treatment of their subjective complaint of HMB. This IUS has been shown to markedly down-regulate hormone receptors in all endometrial compartments (Critchley et al., 1998, Burton et al., 2003), resulting in a "progesterone-deprived" environment. Of the 21 women, 12 returned for a second endometrial sample collection (Figure 11). This tissue was divided and placed in RNA later and NBF as above. The repeat biopsies were paired with pre-insertion samples from the same women for analysis (see Chapter 4). Initial biopsies were dated as proliferative (P) (n=4), early secretory (ES) (n=5), mid secretory (MS) (n=1), late secretory (LS) (n=1) and menstrual (M) (n=1).

2.2 Menstrual blood loss collection

In addition to provision of endometrial tissue, a subset of women (n=81) consented to undergo objective measurement of their menstrual blood loss (MBL) over one menstruation. Of these women, 71 completed the collection and provided an endometrial biopsy (Figure 11). Four women did not complete the menstrual collection, three did not provide endometrial tissue due to unsuccessful sampling and three were excluded due to inconsistencies in endometrial dating. Women were given the same brand of tampon and/or pad (Tampax tampons and Always towels, Proctor and Gamble, UK), with verbal and written instruction on collection (Appendix 3). Measurement of MBL was based on a modified alkaline-haematin method as previously described (Hallberg and Nilsson, 1964, Warner et al., 2004). In brief, used sanitary products were added to a measured volume of 5% sodium hydroxide. The contents were left for 24h to allow conversion of haemoglobin to haematin. During the same time period, a 1 in 200 dilution of the patient's venous blood in 5% sodium hydroxide was made and stored separately. After 24h, an aliquot of sodium hydroxide was removed from the volume soaking the sanitary products and filtered through hardened filter paper (Whartman No. 54, Maidstone, UK). The optical density (OD) of the menstrual blood solution and venous blood sample were then measured using spectrophotometry at 546nm (A_{546}).

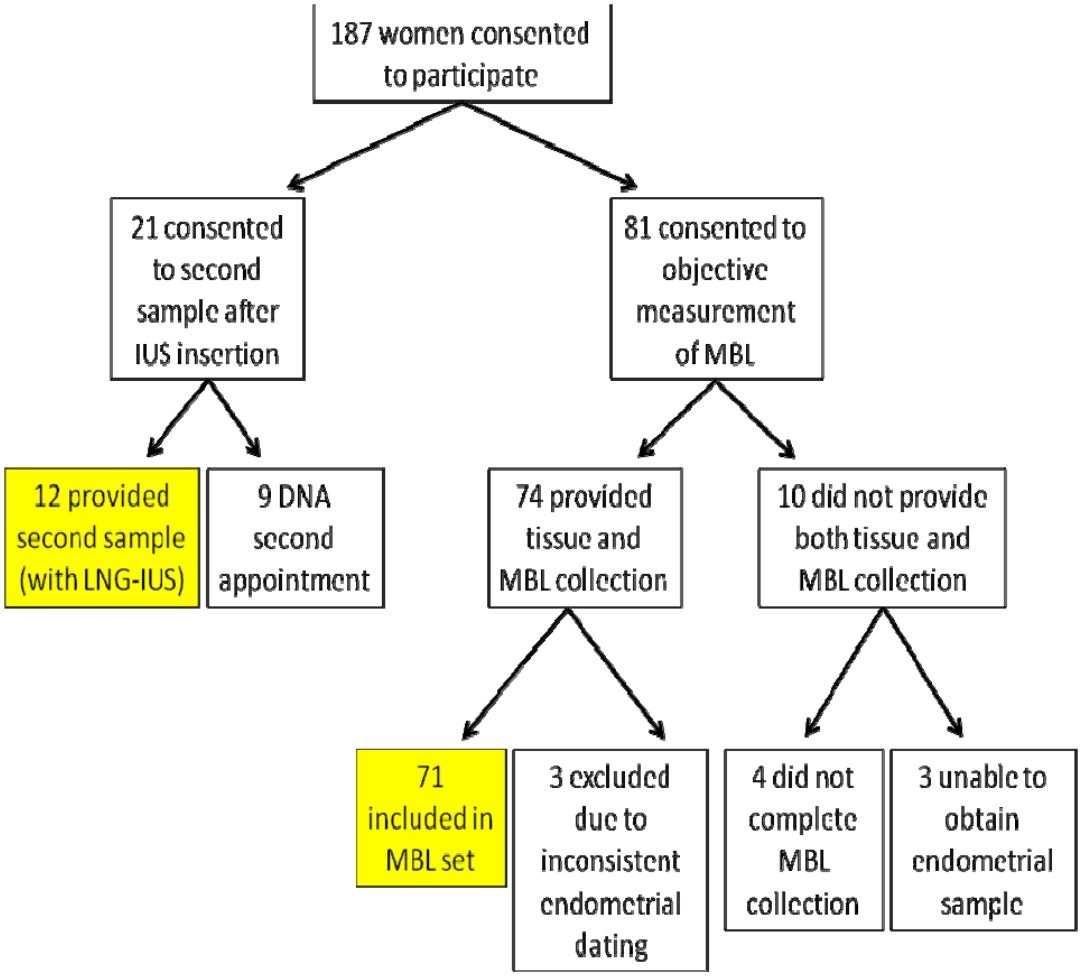


Figure 11. **Recruitment of women to levonorgestrel-releasing intrauterine system (LNG-IUS) group (Chapter 4) and the objectively measured menstrual blood loss (MBL) subset (Chapter 6).** Pre and post LNG-IUS samples are used as an *in vivo* model of progesterone "deprivation". DNA: did not attend.

MBL was calculated as a quantity of the patient's own venous blood using the following equation (van Eijkeren et al., 1986):

$$\text{MBL} = \frac{(\text{OD of menstrual blood solution} \times \text{total volume of added NaOH})}{(\text{OD of venous blood} \times 200)}$$

A MBL measurement of greater than 80ml was classified as heavy menstrual bleeding (HMB) and less than 80ml as normal (NMB). Full details of the endometrial tissue from women with an objective MBL measurement can be found in Chapter 6.2.1, Table 11.

2.2.1 Validation of MBL measurement methodology

Validation of this method was carried out using time expired whole blood measured and applied to the same sanitary products given to the participants. 50ml, 100ml and 300ml of blood was placed onto sanitary products and subject to the same collection and processing procedure as above. The validations were carried out in triplicate for each blood volume. Results showed a consistent 8-9% decrease in MBL value compared to known amount of blood added. Known volume of 50ml = measured value of 46ml (SD 0), 100ml = 92ml (SD 1), 300ml = 273ml (SD 10.5).

2.2.2 Menstrual Pictogram

Thirty women collecting their menstrual loss were also given a menstrual pictogram (see Appendix 4) with verbal and written instructions on mode of completion. Participants were asked to complete the pictogram each time they changed their pad/tampon. The menstrual pictogram contains pictorial representations of graded staining from slight to severely stained sanitary pads and tampons. In addition to scoring the amount of staining, women were asked to specify if pads were normal or night-time and if tampons were regular, super or super-plus. Women were also asked to provide an estimation of extraneous blood loss in the form of clots or flooding. Finally, women indicated if the period they collected was representative of a normal period, or whether it was a lot lighter, slightly lighter, slightly heavier or a lot heavier than usual.

A scoring system for the feminine hygiene product pictogram was devised (see Appendix 5) based on previous studies (Higham et al., 1990, Wyatt et al., 2001, Wilkens et al., 2008). In addition, extraneous loss was scored separately. Small, medium and large clots that were documented were scored as 1, 3 and 5ml respectively and each episode of flooding was assigned 3ml. The scores from the menstrual pictogram icons for feminine hygiene products (without extraneous loss) were compared with objective blood loss measured by the alkaline-haematin technique (results described in Chapter 6.3.1).

2.3 *In vitro* culture

2.3.1 Endometrial explant culture

Endometrial biopsies were divided into equal explants and incubated on raised platforms in 24 well plates, just covered with serum free RPMI 1640 medium plus penicillin (50 μg/ml, Sigma, Dorset, UK), streptomycin (50μg/ml, Sigma) and gentamycin (5μg/ml, Sigma). Experimental conditions and treatments are detailed within each results chapter (Chapter 4.2.3 and Chapter 6.2.10).

2.3.2 Isolation and culture of primary human endometrial stromal (HES) cells

Stromal cells were isolated from human endometrial tissue from the proliferative, early and mid secretory phases by enzymatic digestion previously described (Kane et al., 2008). Briefly, specimens were washed in Dulbecco's PBS (Sigma) and finely dissected with a scalpel. The chopped tissue was digested in collagenase (10mg/ml, Sigma) and DNase (4mg/ml, Sigma) for 90 min at 37ºC. The tissue was resuspended in 2ml RPMI 1640 medium (Sigma) and repeat passage through a 19-gauge needle was used to aid tissue dispersion. Filtration through a 40μm nylon cell strainer (Falcon; BD Biosciences, Bedford MA, USA) was carried out prior to centrifugation (1700rpm, 3 min). The supernatant was discarded and the pellet resuspended in 10 ml RPMI 1640 medium supplemented with 10% fetal calf serum (Mycoplex; PAA Laboratories, Kingston-Upon-Thames, UK), penicillin (50μg/ml, Sigma), streptomycin (50μg/ml, Sigma) and gentamycin (5μg/ml, Sigma). The cell suspension was then plated into a 162cm^3 flask and grown to confluence. All

experiments were carried out at passage ≤4 in serum free RPMI at timepoints identified by timecourse experiments.

2.3.3 Transfection and culture of endometrial epithelial cells

Human Ishikawa endometrial adenocarcinoma cells (European collection of cell culture, Centre for Applied Microbiology, Wiltshire, UK) had been previously stably transfected as described in (Sales et al., 2004), with either the prostaglandin F-series prostanoid receptor (FPS) or the prostaglandin E-series prostanoid receptor 2, one of the G protein-coupled receptors shown to transduce prostaglandin E_2 signalling in the human endometrium (EP2S cells). Cells were maintained in DMEM nutrient mixture F-12 with glutamax-1 and pyridoxine, supplemented with 10% fetal calf serum, 1% antibiotics (stock 500IU/ml penicillin and 500μg/ml streptomycin), and 200μg/ml G418 (Calbiochem, Nottingham, UK) at 37ºC. All experiments were carried out in serum free DMEM media and are described in further detail within each results chapter (Chapter 4.2.2, Chapter 5.2.2-4 and Chapter 6.2.11).

2.4 RNA extraction and quantitative reverse transcription polymerase chain reaction

2.4.1 RNA extraction

Total RNA from cells and endometrial tissue was extracted using the commercially available product RNeasy Mini Kit (Qiagen Ltd, Sussex, UK) according to the manufacturer's instructions. Samples were treated for DNA contamination by DNAase digestion during RNA purification. Following extraction, RNA was quantified using the Nanodrop 1000 spectrophotometer v3.7 (ThermoScientific, DE, USA) and stored at -80°C. Quality of the RNA was assessed using the Agilent 2100 Bioanalyser system in combination with the RNA6000 nano chips (Agilent Technologies, Cheshire, UK), according to manufacturer's instructions. Only RNA that displayed intact 18S and 28S peaks was reverse transcribed to cDNA for real-time PCR analysis. In addition, the RNA Integrity Number (RIN) was determined by the 2100 Expert software (Agilent Technologies) for tissue samples sent for array analysis. A RIN number below 7.5 suggests degradation of the RNA; all array samples had a RIN above 9.5.

2.4.2 Reverse transcription

RNA samples were reverse transcribed using $MgCl_2$ (5.5mM), deoxy (d) nucleotide triphosphates (0.5mM each) random hexamers (2.5µM), ribonuclease inhibitor (0.4U/µl) and multiscribe reverse transcriptase (1.25U/ µl; all from PE Biosystems, Warrington, UK). The mix was aliquoted into individual tubes (8µl per tube), and 200ng of RNA added (2µl per tube of 100ng/µl RNA). A tube with no reverse transcriptase and a further tube with water replacing RNA were included to control for DNA contamination. After mixing, samples were incubated for 20 min at 25ºC, 60 min at 42ºC, and 5 min at 95ºC. Complementary DNA (cDNA) samples were subsequently stored at -20ºC.

2.4.3 Quantitative polymerase chain reaction (Q-RT-PCR)

Q-RT-PCR was used to measure the level of gene specific sequences in cDNA generated by reverse transcription. This method uses forward and reverse primers to the target sequence of cDNA, along with a probe that recognizes a sequence between the two primer annealing sites. The probe is labelled with two fluorescent dyes; a 5' reporter label (FAM; 6-carboxyfluorescein) and a 3' quencher label (TAMRA; 6-carboxytetramethylrhodamine). When the probe is intact, the quencher suppresses the fluorescence of the reporter. During the amplification phase of the PCR process, the probe is cleaved by AmpliTaq Gold. This separates the two fluorescent labels, removing the inhibition of the reporter label. The resulting fluorescent signal is directly proportional to the amount of PCR product. Fluorescence is only detected if the target sequence for the probe is amplified during the reaction, preventing the detection of non-specific amplification. 18S ribosomal RNA detection was used as an internal control as levels remain constant relative to the amount of cDNA present. The reporter dye present on the 18S probe is VIC, which emits fluorescence at a different wavelength to the FAM reporter on the probe of the gene of interest.

Primers and probes used in this study were designed using Primer Express Software (Applied Biosystems, Warrington, UK) or the Universal Probe Library Assay Design Centre (Roche Diagnostics Ltd, Burgess Hill, UK). BLAST (basic local alignment search tool) was used to determine the presence of sequences in the scientific

databases that are similar to those amplified by specific primers. In all cases, primer and probe sets for target genes of interest were found to be unlikely to amplify non-specific templates.

A reaction mix was prepared containing Taqman buffer (5.5mM $MgCl_2$, 200µM dATP, 200µM dCTP, 200µM dGTP, 400µM deoxyuridine triphosphate), ribosomal 18S primers and probe (Applied Biosystems, Warrington, UK) and specific forward primer, reverse primer and probe for the gene of interest (Table 3). After mixing, 36µl aliquots were placed in separate tubes and 1.5µl cDNA added, including water negative and reverse transcriptase negative cDNA samples. Into one aliquot, 1.5µl water was added as a no template control. Triplicate 12µl samples were placed into a PCR plate. PCR was carried out using an ABI Prism 7900 (Applied Biosystems) using standard conditions. Data were analysed and processed using Sequence Detector version 2.3 (PE Biosystems).

PCR data was analyzed using the $\Delta\Delta C_T$ method as described by Applied Biosystems. This normalises the amount of target mRNA to RNA loading for each sample using 18S ribosomal RNA, which is then related to an internal control. The linearity of the response of primers and probes to specific cDNA was determined by serial dilution. Primers and probes were diluted up to 64 times using a standard pool of RNA. The log of ng total RNA was plotted against ΔC_T units and the gradient of the lines through these points determined. The absolute value of the slope was <0.1 for all primers and probes used herein.

Table 3. Taqman Primers and Probes

Gene	Accession No.	Forward Primer	Reverse Primer	Primer Conc. (nM)	Probe	Probe Conc. (nM)
VEGF	NM_001171630	TACCTCCACCATGCCAAGT	TAGCTGCGCTGATAGACAT	300	ACTTCGTGATGATTCTCC	200
KDR	NM_002253	Assay on Demand Hs00176676_m1	N/A		N/A	
CTGF	NM_0019101.2	TGCACCGCCAAAGATGGT	GGCACGTGCACTGGTACTTG	300	CTCCCTGCATCTTCGGTGGTACGGT	200
IL-8	NM_000584.2	CTGGCCGTGGCTCTCTTG	TTAGCACTCCTTGGAAAACTG	300	CCTTCCTGATTTCTGCAGCTCTGTGTGAA	200
CXCR2	NM_001168298.1	TGCTCTTCTGGAGGTGTCCTACA	AGATCTTCACCTTTCCAGAAATCTTC	300	CCCAGCGACCCAGTCAGGATTTAAGTTT	200
ADM	NM_001124.1	TTCCGTCGCCCTCATGTAC	CCACTTATTCCACTTCTTTCGAAAC	300	TGGGTTCGCTCGCCTTCCTAGGC	200
CLR	NM_005795.4	GGACTCAATTCAGTTGGGAGTTACTAG	GAGCCATCCATCCCAGGTT	300	CCCCATTCAACAAGCAGAAGGCGTTT	200
RAMP1	NM_005855.2	AATGCAGAGGTGGACAGGTTCT	CGGCCCTGCCTGAGATG	300	CCTGGCAGTGCATGGCCGCTAC	200
RAMP2	NM_005854.2	CGAGATTGCCTGGAGCACTT	TCTGGTGAGTCTCAAAAGATGATCCT	300	CAGAGTTGTTTGACCTGGGCTTCCCC	200
RAMP3	NM_005856.2	CCGAGTTCATCGTGTACTATGAGAGT	GTGGATGCCGGTGATGAAG	300	TGCTACTGGCCCAACCCCCTGG	200
ET-1	NM_001955.3	TCTCTGCTGTTTGTGCTTG	GAGCTCAGCGCCTAAGTCTG	200	Universal probe library: 50	100
ET_B	NM_000115	CTTGGAGTCTGGACATCTGAAA	CTGCATGCTGCTACCTGCT	200	Universal probe library: 21	100

Gene	Accession No.	Forward Primer	Reverse Primer	Primer Conc. (nM)	Probe	Probe Conc. (nM)
bFGF	NM_002006.4	TTCTTCCTGCGCATCCAC	TTCTGCTTGAAGTTGTAGCTTGAT	200	Universal probe library: 7	100
Ang-2	NM_001147.2	GCCGCTCGAATACGATGACT	ATTAGCCACTGAGTGTTGT	300	TTCTCCAGCACTTGCAGCC	200
HIF-1α	NM_001530.3	CGCATCTTGATAAGGCCTCTGT	AATCACCAGCATCCAGAAGTTTC	300	TCACACGCAAATAGCTGATGGTA AGCCTCAT	200
HIF-1β	NM_014862.3	CCCGGTCAGACTGAAGTGTT	TCCCCTGGGTTGGATCTC	200	Universal probe library: 44	100
HIF-2α (EPAS1)	NM_001430.3	AATCAGCTTCCTGCGAACAC	GCTTCGGACTCGTTTTCAGA	200	Universal probe library: 55	100
ESM-1	NM_007036.3	Assay on Demand Hs00199831_ml	N/A		N/A	
CXCR4	NM_003467.2	CTGTTGAGCAGAGGGTCCAG	ATGAATGTCCACCTCGCTTT	200	Universal probe library: 55	100
HEY1	NM_001040708.1	CATACGGCAGGAGGGAAAG	GCATCTAGTTCTTCAATGATGCT	200	Universal probe library: 29	100
SMAD3	NM_005902.3	Assay on Demand Hs00969210_m1	N/A		N/A	
ACTG2	NM_001615.3	Assay on Demand Hs01123712_m1	N/A		N/A	
PNKD	NM_015488.4	Assay on Demand Hs00737773_m1	N/A		N/A	
IDH1	NM_005896.2	Assay on Demand Hs00271858_m1	N/A		N/A	
LMNA	NM_005572.3	AGCAAAGTGCGTGAGGAGTT	AGGTCACCCTCCTTCTTGGT	200	Universal probe library: 17	100

2.5 Western Blotting

Western blotting identifies the presence of proteins by detection with specific antibodies after separation by electrophoresis and transfer to a polyvinylidene difluoride membrane.

2.5.1 Nuclear protein extraction from cells

Cytoplasmic proteins were extracted with a cytoplasmic protein lysis buffer [10mM HEPES (pH 7.8), 10nM KCl, 2mM $MgCl_2$, 1mM dithiothreitol, 0.1mM EDTA, 10% Nonident P-40] containing protease inhibitors (Complete mini protease inhibitor cocktail; Roche Diagnostics Ltd., Lewes, UK). The membrane fraction was pelleted by centrifugation at 13000rpm for 1 min at 4ºC. The cytoplasmic fraction supernatant was removed and stored at -80ºC. Thereafter, the nuclear fraction was extracted with a nuclear protein lysis buffer [50mM HEPES (pH 7.8), 50nM KCl, 300mM NaCl, 0.1mM EDTA, 1mM dithiothreitol, 10% glycerol] containing protease inhibitors (Roche) followed by agitation for 20 min at 4ºC and centrifugation at 13000 rpm for 5 min at 4ºC. The nuclear fraction supernatant was removed and stored at -80ºC. Protein content was determined using protein assay kits (Bio-Rad, Hemel Hempstead, UK).

2.5.2 Nuclear protein extraction from endometrial tissue

Endometrial tissue was incubated in RNA later solution (Ambion Ltd) for 24h and subsequently frozen at -80ºC. Nuclear protein was extracted from approximately 20mg of this frozen tissue using a commercially available nuclear extract kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions.

2.5.3 Electrophoresis

For HIF-1α and β-actin, a total of 10μg of nuclear protein was resuspended in a 2:1 ratio with Laemmli buffer [125mM Tris-HCl (pH 6.8), 4% SDS, 5% 2-mercaptoethanol, 20% glycerol and 0.05% bromophenol blue] and denatured for 5 min at 90ºC. Proteins were separated on 4-12% Bis-Tris Gels (NuPAGE Novex, Invitrogen, Carlsbad, CA) in NuPage MOPS SDS running buffer (Invitrogen),

alongside Precision Plus protein standards, all blue (Bio-Rad, Hemel Hempstead, UK). The electrophoresis tank was run at 150V, 50mA, 25W for 70min.

2.5.4 Electrotransfer

The protein was transferred onto polyvinylidene difluoride membranes (Millipore, Watford, UK), which had been pre-soaked in methanol. Transfer buffer: 12.2g tris (Sigma), 5.6g glycine (Sigma), 200ml methanol (Fisher Scientific), 1800ml distilled H_2O. The gel and membrane were assembled in the electrotransfer apparatus and the transfer performed at 250mA, 300V, 50W for 2h.

2.5.5 Western Blot

Membranes were blocked overnight in 5% milk solution in Tris buffered saline with Tween (TBST; 50mM Tris-HCl, 150mM NaCl and 0.05% vol/vol Tween 20). After washing with TBST, the membranes were incubated with primary antibodies for 2h at room temperature; mouse monoclonal anti-HIF-1α antibody (BD Biosciences, Oxford, UK) diluted 1:250, rabbit polyclonal to β-actin (Abcam, Cambridge, UK) at 1:5000. After washing, the membrane was incubated with horseradish peroxidase conjugated goat anti-mouse IgG (Dako) or horseradish peroxidase conjugated mouse anti-rabbit IgG (Sigma), both at 1:20000. The chemiluminescent horseradish perioxidase substrate (Immobilon Western, Millipore Corporation, MA, USA) was used for immunoreactive protein detection according to manufacturer's instructions.

2.6 Immunohistochemistry

This technique utilises specific primary antibodies to localise an antigen within a section of tissue. Immunohistochemistry was performed to detect IL-8, VEGF, CTGF, AM, HIF-1α and HIF-1β proteins in endometrial tissue biopsies. With the exception of HIF-1α, all antigens were detected using a standard avidin-biotin horseradish peroxidase (ABC-HRP) method. A sequence of primary antibody, biotinylated secondary antibody and the pre-formed avidin-biotin-HRP enzyme complex is used in this method. Chain polymer-conjugated technology was employed for HIF-1α staining, using the EnVision system. Here the primary antibody is followed by incubation with an enzyme-labelled inert molecule of

dextran, meaning avidin and biotin blocking steps were not required. Both methods utilised 3,3-diaminobenzidine for detection.

All protocols were optimised to determine appropriate conditions for maximal and specific immunostaining. Specific protocols are summarised in Table 4. All immunohistochemical staining was performed on paraffin sections (3μm). These were dewaxed for 10min in xylene before rehydration in descending grades of alcohol. Slides were washed in distilled water for 10min prior to antigen retrieval.

2.6.1 Antigen retrieval

Different antigen retrieval methods were employed depending on the antibody used (see Table 4).

- A pressure cooker was filled with 2 litres of 0.01M sodium citrate buffer (pH 6) and placed on a hot plate. When heated, slides were submerged and the lid sealed. Slides were heated for 5min at full pressure.
- Microwave antigen retrieval was carried out in either pH 6 antigen retrieval buffer (Vector Laboratories, Peterborough, UK) or pH 9.0 Hiar buffer (10 mM TRIS; 1 mM EDTA; and 0.05% Tween-20). Sections were heated in the appropriate buffer on full power for 15min.

All slides were allowed to cool for 20min before washing in PBS for 10min.

2.6.2 Blocking

Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 10min at room temperature and washed twice in PBS. Slides were incubated sequentially with avidin and biotin (Vector Labs, Peterborough, UK) for 10min at room temperature to block endogenous avidin-binding activity in all but HIF-1α staining. After washing in PBS, background staining was further prevented by incubation with a non-immune block to prevent non-specific binding of the secondary antibody. Serum from the species in which the secondary antibody was raised was diluted 1:5 in PBS with 5% BSA (Sigma-Aldrich Company Ltd, Dorset, UK). Alternatively, the commercially available protein block (DAKO, Cambridge, UK) was used.

Table 4. Summary of immunohistochemical protocols

Protein	Antigen retrieval	Non-immuno block serum	Primary antibody (1°)	Dilution of 1°	Negative Control	Secondary antibody (2°)	Dilution of 2°
VEGF	microwave pH 9	Dako protein block	Mouse monoclonal (R&D Systems, Abingdon, UK)	1 in 80	Mouse IgG	Horse anti-mouse (Vector Labs, Peterborough, UK)	1 in 200
CTGF	microwave pH 6	Dako protein block	Goat polyclonal (Santa Cruz, CA95060, USA)	1 in 100	Peptide (Santa Cruz)	Horse anti-goat (Vector)	1 in 200
AM	microwave pH 6	Goat	Rabbit polyclonal (Phoenix Pharmaceuticals Inc, Karlsruhe, Germany)	1 in 1000	Rabbit IgG	Goat anti-rabbit (Vector)	1 in 200
HIF-1α	microwave pH 6	Dako protein block	Rabbit polyclonal (Santa Cruz)	1 in 100	Anti-Rabbit Envision	N/A	N/A
HIF1β	pressure cooker	Goat	Mouse monoclonal (Abcam, Cambridge, UK)	1 in 1000	Mouse IgG	Goat anti-mouse (Vector)	1 in 200
IL-8	None	Goat	Rabbit polyclonal IL-8 (0427)	1 in 750	Rabbit IgG	Goat anti-rabbit (Vector)	1 in 300

2.6.3 Primary Antibodies and controls

Tissue sections were incubated overnight at 4°C with 125µl of primary antibody at the appropriate concentration. Dilutions were carried out in the serum solution used for blocking or in commercially available antibody diluent (DAKO). Where possible, the primary antibody was pre-absorbed against a blocking peptide for use as a negative control. Alternatively, the primary antibody was substituted with an equimolar concentration of generic immunoglobulins from the same species in which the primary antibody was produced. Positive controls were tissue sections known to express high levels of the protein of interest. The next day, sections were washed between each stage with PBS containing 0.05% Tween-20.

2.6.4 Secondary Antibodies

Sections were incubated with the appropriate biotinylated secondary antibody, raised against the immunoglobulins of the species in which the primary antibody was produced. Antibodies were diluted (see Table 4) and 125µl applied to all sections for 1h at room temperature. For HIF-1α immunohistochemical staining, EnVision polymer reagent was used in place of the secondary/tertiary antibody (Figure 13B).

2.6.5 Amplification

The ABC-Elite detection system (Vector) was used to amplify the signal from the biotinylated secondary antibody. Three drops of ABC-Elite were added per slide and incubated for 1h at room temperature. This step was not required when Envision was used (HIF-1α protocol).

2.6.6 Detection

Positive staining was visualised using the peroxidase substrate 3,3-diaminobenzidine (DAB; DAKO), which forms a brown precipitate on contact with the antigen-antibody complex.

2.6.7 Dehydration and mounting

All slides were washed in distilled water before counterstaining with Harris' haematoxylin. Sections were then dehydrated in ascending grades of alcohol, incubated for 10min in xylene and mounted in Pertex.

2.6.8 Semi-quantitative scoring

Localisation and intensity of immunostaining was evaluated blindly by two independent observers using a semi-quantitative scoring system. The intensity of staining was graded with a three point scale (0 = no staining, 1 = mild staining, 2 = strong staining). This was applied to the glands and stromal cells in both the basal and functional layer of the endometrium (in full thickness sections), as well as the surface epithelium, perivascular and endothelial cells where visualised. The percentage of tissue in each intensity scale was recorded. A value was derived for each of the cellular compartments by using the sum of these percentages after multiplication by the intensity of staining. This histoscore system is a standard method used in previous studies (Aasmundstad et al., 1992, Wang et al., 1998a, Critchley et al., 2006b) and has previously been validated in a set of tissue sections where immunoreactivity was also measured with a computerised image analysis system (Wang et al., 1998b). There was a strong correlation between quantitative data derived from image analysis and subjective scores by trained observers.

2.7 Enzyme linked immunosorbant assay (ELISA)

This technique is used to determine the concentration of a given substance in a solution by comparison with a standard curve created from solutions of known concentration. There are two principle forms of ELISA:

(i) Sandwich ELISA. Plates are pre-coated with an antibody (C), which binds to a specific epitope on the substance to be measured (S). Next a detection antibody (D) that is labelled with biotin, binds to a different epitope of S. Finally, streptavidin peroxidase binds to biotin and is detected by addition of substrate. This type of ELISA was used to measure IL-8, CTGF and VEGF secreted proteins.

(ii) Competition ELISA. Plates are pre-coated with the recombinant protein of interest (rP) and then blocked to remove non-specific binding. The solution containing the protein of interest (P) is then added in combination with a primary anti-serum. This antiserum recognises identical epitopes on rP and P and competitively binds. If the solution being measured contains a high concentration of P, then most of the antiserum will have become conjugated to it and will be washed off in subsequent wash cycles. In contrast, if the solution contains low concentrations of P, then the antibody will have become bound to rP on the ELISA plate. A secondary antibody is added and detected. Sample wells with low levels of P (high levels of antibody bound to rP) will have a high OD value and those with high levels of P (low amounts of antibody bound to rP) will have a low OD value. This form of ELISA was used to measure AM protein.

2.7.1 IL-8 ELISA

IL-8 protein concentrations in culture supernatants was quantified using a commercially available enzyme linked immunosorbent assay (ELISA) (R&D Systems, Oxford, UK) as described previously (Denison et al., 1998). Plates were coated overnight with 100µl/well of mouse monoclonal anti-human IL-8 capture antibody (500µg/ml diluted 1:360 with ELISA buffer) (R&D Systems). After washing, 100µl of standard or sample was added. A biotinylated polyclonal goat anti-human IL-8 detection antibody (50µg/ml diluted 1:1200) was used (R&D Systems). 100µl/well of strepavidin peroxidase (1:2000 dilution) was added and plates incubated for 30min at room temperature. After washing, 200µl Tetra Methyl Benzidine substrate was added to each well for 10min, followed by quenching with 50µl/well of 2N sulphuric acid. Plates were read on a plate reader at 450nm. The intra- and interassay precisions were 9.1% and 22% respectively and the detection limit of the assay was 8 pg/ml.

2.7.2 CTGF ELISA

CTGF secreted protein concentrations were measured using a human CTGF ELISA development kit (PeproTech, London, UK) according to manufacturer's instructions. In brief, 96 well ELISA microplates were coated with 100µl of rabbit anti human CTGF capture antibody at 1µg/ml and incubated overnight. After washing, wells were blocked for 1h with 1% BSA in PBS. 100µl of sample or standard was added per well and incubated at room temperature for 2h. A biotinylated antigen-affinity purified rabbit anti human CTGF detection antibody was used at 100µg/ml. Incubation with avidin-horseradish peroxidase (HRP) conjugate at a dilution of 1:2000 for 30min was followed by detection with ABTS liquid substrate solution (Sigma). Colour development was measured on an ELISA plate reader at 405nm. Minimum Detectable Concentration = 63 pg/ml. There no significant cross-reactivity for human BMP-4, CTGFL/WISP-2, CYR61, IGF-I, IGF-II, IGF-BP1, NOV, TGF-β, WISP-1 or WISP-3.

2.7.3 VEGF ELISA

The human VEGF ELISA development kit (PeproTech, London, UK) was used according to manufacturer's instructions to measure VEGF protein concentrations in culture supernatants. Each well of the ELISA plate was coated with 100µl of 0.5µg/ml rabbit anti human VEGF capture antibody. The next day, the plate was washed, blocked for 1h and incubated with 100µl of sample or standard per well. After washing, 100µl of 0.25µl/ml of biotinylated rabbit anti human VEGF detection antibody was added to each well and incubated for 2h. Incubation with avidin-HRP conjugate at a dilution of 1:2000 for 30min was followed by detection with ABTS liquid substrate solution (Sigma). Colour development was measured on an ELISA plate reader at 405nm. Minimum Detectable Concentration = 63 pg/ml. There was 100% cross-reactivity for human $VEGF_{121}$ and murine VEGF but no significant cross-reactivity for human EG-VEGF, EGF, RANTES, FGF-16, GM-CSF, PDGF-AA, PDGF-AB, PDGF-BB or SCF.

2.7.4 AM ELISA

AM concentration in conditioned media was measured using a human AM (1-52) EIA kit from Phoenix Peptides (Karlsruhe, Germany) according to manufacturer's instructions. 50µl of standards, samples and positive controls were added to each well with 25µl of anti-serum (rabbit anti-peptide IgG) and 25µl of biotinylated peptide. The plate was incubated at room temperature for 2h. After washing, 100µl of streptavidin-HRP solution was added per well and incubated for 1h. 100µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to each well after washing and incubated for 1h. Reactions were terminated with 2N HCl before absorbance optical densities were read at 450nm. Minimum Detectable Concentration = 0.13 ng/ml, interassay error <14%, intraassay error <5%. There was 100% cross-reactivity for rat AM (1-50) and human AM (13-52) and mouse AM (1-50) but no reported cross-reactivity for endothelin-1, amilin, CGRP, CGRP-2, αANP or BNP-32.

2.8 Capillary tube formation assay

A capillary tube formation assay was used to assess *in vitro* angiogenic potential, as endothelial proliferation and migration are integral components of angiogenesis. Methodology was previously established in our laboratory (Rae et al., 2009). 100µl Matrigel (BD Biosciences, Bedford, MA) was added per well in a 48 well plate and allowed to polymerise for 1h at 37ºC. Human umbilical vascular endothelial cells were seeded at a density of $2x10^4$ in 200µl EBM-2 media (Lonza, MD, USA) supplemented with GA1000 and ascorbic acid SingleQuots®. 200µl of treated RPMI media or culture supernatants from endometrial cell or explants experiments were then added to the HUVEC cells (see Chapter 3.2.5 and Chapter 6.2.11). Each supernatant was assessed in triplicate in three separate experiments. Capillary tube formations were visualised after 8h. Images were captured in the same position in each well using an inverted microscope at x5 magnification. Branch points of the formed tubes were counted by an observer blinded to the sample origin and an average of the replicates was determined after unblinding.

2.9 RNA Interference

This "loss of function" technique is used to elucidate the function of a gene. RNA interference (RNAi) occurs naturally *in vivo,* as part of a cell's antiviral defence mechanism (Fire et al., 1998). Double stranded RNA is cleaved into short interfering RNAs (siRNA) by an enzyme known as Dicer. These siRNAs then become integrated into a protein complex named the RNA induced silencing complex (RISC). This guides the siRNA to its target RNA and becomes involved in the degradation of this complementary sequence to suppress gene expression. Production of synthetic siRNA sequences has allowed this method to be harnessed as a tool for assessing gene function (Figure 12).

2.9.1 Small interfering RNA

Double stranded, chemically synthesized RNA that was chemically labelled with 6-FAM was used as a transfection indicator in EP2S cells (siGLO Green, Thermo Scientific Dharmacon®, Lafayette, CO). Approximately $1x10^5$ EP2S cells were seeded per well of a 6 well plate in DMEM media containing 10% fetal calf serum. The next day, when cells were at 60% confluence, transfection mixes were prepared containing siGLO and HiPerfect transfection reagent (Qiagen, West Sussex, UK). This mix was swirled gently and incubated for 10 min before dropping onto the cells. SiGLO was added at a final concentration of 0, 5, 10, 20, 50 and 100nM. HiPerfect was added at 1:100, 1:200 and 1:300 for each concentration of siGLO. EP2S cells were incubated with transfection mix for 8h at 37ºC before washing in PBS and incubating with DMEM plus 10% FBS for 24h. Cells were then incubated with serum free DMEM overnight before checking transfection efficiency using the Axiovert 200 microscope (Zeiss, Welwyn Garden City, UK).

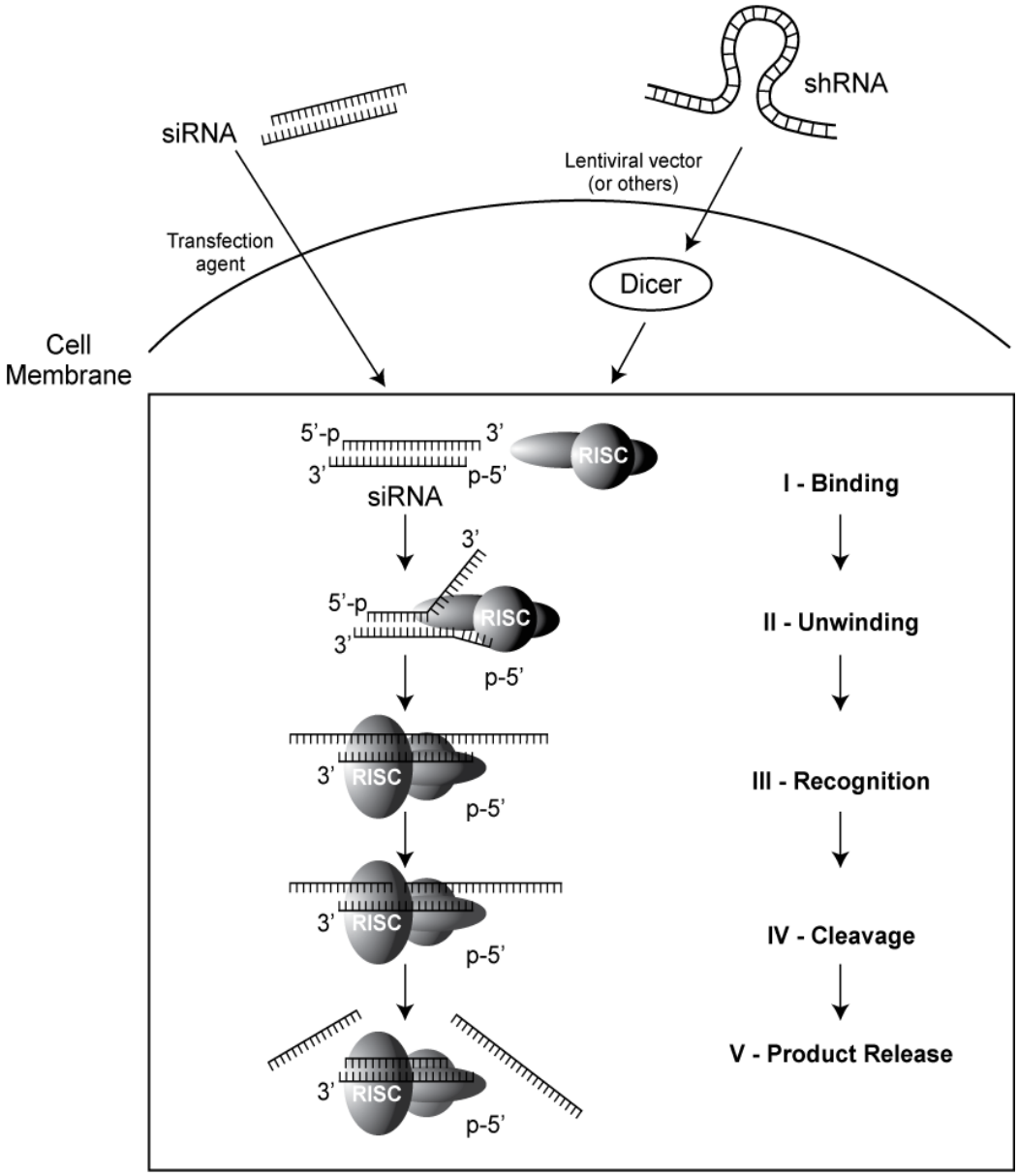


Figure 12. **The mechanism of RNA interference**. Short interfering RNA (SiRNA) constructs enter the cell with the assistance of cationic and neutral lipids. Short hairpin RNA (ShRNA) constructs can be transfected into cells using a vector, e.g. lentivirus. These ShRNA constructs are then converted to SiRNA by endogenous RNase III-like enzyme (Dicer). SiRNA sequences interact with the RNA induced silencing complex (RISC) to form single strand sequences which recognise and bind to complementary RNA sequences. Cleavage of target sequences prevents their transcription, leading to decreased protein production and "silencing" of gene function.

2.9.2 Short hairpin RNA

RNA polymerase III can be used to drive individual transcripts to adopt stem-loop structures, known as short hairpin RNA (ShRNA) sequences. ShRNA can be transfected into a cell by means of a vector and are then processed into siRNAs by the RNAi machinery. Two short hairpin RNA (ShRNA) sequences against human HIF-1α and a scrambled control oligonucleotide (TIB MOLBIOL) were kindly donated by Professor T Cramer (Charité-Universitätsmedizin Berlin, Germany). Two different 19-nucleotide (nt) sequences derived from human HIF-1α mRNA (U22431; bp 1470-1489 and bp 2192-2211) were used and have been described previously (Mizukami et al., 2004, Sowter et al., 2003). These constructs are termed HIF-1α/shRNA1470 and HIF-1α/shRNA2192. Approximately 6 x 10^4 Ishikawa endometrial epithelial cells (EP2S and FPS) were seeded per well in a 12 well plate. The next day cells were transiently transfected with lentivirus at a multiplicity of infection (MOI) of 10 for 24h. Cells were incubated in serum free media overnight before treatment with 100nM PGE_2 , 100nM $PGF_{2\alpha}$ or placement in the hypoxic chamber for 8h. Cells were washed with PBS and harvested, and RNA or protein extracted for PCR or Western blot analysis.

2.10 Statistical analysis

All statistical analysis was carried out using GraphPad Prism Software (GraphPad Prism Software, Inc., San Diego, CA). Details of specific statistical tests used for each data set are included in the relevant chapter (Chapter 3.2.6, 4.2.7, 5.2.10 and 6.2.12). In brief, comparison of two data sets was performed using a student's t-test (if normally distributed) or Mann Whitney test (if not normally distributed). Comparison of multiple groups with one grouping variable were compared with one way ANOVA or Kruskal Wallis tests, with application of appropriate post-test multiple comparison analyses. Comparison of multiple data sets with two grouping variables was carried out using a two way ANOVA. All graphs display mean ± standard error of mean, unless otherwise stated in figure legends.

3. Endometrial Repair Factors across the Menstrual Cycle

3.1 Introduction

Throughout the reproductive years of a woman's life the endometrium is cyclically subjected to inflammation and tissue breakdown during menstruation. It has a remarkable capacity for repeated, scar-free repair without loss of function. The factors involved in this efficient endometrial repair remain undefined. The processes involved are likely to include inflammation and its timely resolution, tissue formation, tissue remodelling and angiogenesis (detailed in Chapter 1). Hence, factors known to be involved in these processes elsewhere in the body have a putative role in endometrial repair.

IL-8 is a cytokine present in the epithelial and perivascular cells of the human endometrium (Arici et al., 1998, Jones et al., 2004, Critchley et al., 1994). It is best known for its potent chemotaxis of neutrophils, T-cells and basophils (Larsen et al., 1989, Mukaida et al., 1989) and mediates its actions via CXCL1/2 receptors. Neutrophil depletion in the mouse model of simulated menstruation resulted in a marked delay in endometrial repair, signifying the importance of an inflammatory response for efficient repair (Kaitu'u-Lino et al., 2007b). In addition, IL-8 secreted by human synovial tissue has been shown to have angiogenic properties (Koch et al., 1992) and *in vitro* IL-8 stimulated proliferation of aortic vascular smooth muscle cells (Yue et al., 1994). The inflammatory, angiogenic and mitogenic effects of IL-8 make it an attractive candidate for mediating endometrial repair.

The angiogenic properties of vascular endothelial growth factor (VEGF) and adrenomedullin (AM) are well established (Carmeliet, 2005, Ferrara, 2004, Nayak and Brenner, 2002, Shindo et al., 2001, Nikitenko et al., 2000) and have been discussed previously (Chapter 1.3.5.3). Both have been identified in human endometrial epithelial, endothelial and stromal cells (Charnock-Jones et al., 1993, Zhao et al., 1998) and are therefore putative endometrial repair factors. VEGF acts through two transmembrane tyrosine kinase receptors, VEGF receptor 1 (VEGFR1/Flt1) and VEGF receptor 2 (VEGFR2/Flk1/KDR), both of which are expressed in the human endometrium (Nayak et al., 2000, Punyadeera et al., 2006). Although VEGF binds with high affinity to both receptors, most of its biological

effects appear to be mediated by VEGF receptor 2 (Li et al., 2002). The complexities of AM signalling have also been delineated. AM acts through a G protein coupled receptor known as the calcitonin receptor like receptor (CLR). CLR can act as either a calcitonin gene-related peptide receptor or an AM receptor. Receptor activity modifying proteins (RAMPs) associate with CLR to determine its ligand binding specificity (Sexton et al., 2001). RAMP2 and RAMP3 association promotes binding of AM to CLR, while RAMP1 and CLR association promotes binding of the calcitonin gene-related peptide (McLatchie et al., 1998).

CTGF is a member of a new family of structurally related proteins that have angiogenic, wound healing and diverse cellular functions (Brigstock, 2002). The angiogenic properties of CTGF have been demonstrated *in vitro* and *in vivo* (Shimo et al., 1999). In addition, increased levels of CTGF have been observed in areas of tissue injury (Igarashi et al., 1993, Schwab et al., 2001), leading to speculations that CTGF is involved in tissue repair. Therefore, CTGF has a potential role in initial endometrial repair and subsequent tissue remodelling.

ET-1 is a pleiotropic molecule, best known for its actions as a potent vasoconstrictor (Masaki, 2004). It acts through two heptahelical G protein-coupled receptors ET_A and ET_B (Sakamoto et al., 1991, Hosoda et al., 1991). Both ET_A and ET_B mRNA has been identified in the human endometrium (O'Reilly et al., 1992). ET-1 has also been shown to act as a mitogenic and pro-angiogenic factor in cancer cells (Grant et al., 2003). In addition, it may have the ability to modulate inflammatory responses. ET-1 increased IL-6 production by rat stromal cells (Agui et al., 1994) and increased neutrophil adhesion to bovine endothelial cells *in vitro* (Lopez Farre et al., 1993). Hence ET-1 may not only contribute to vascular vasoconstriction at the time of menstruation, but may also contribute to endometrial repair.

Electron microscopic and hysteroscopic analysis of the endometrial cavity has revealed that endometrial repair takes place during the menstrual phase of the cycle (Ludwig and Spornitz, 1991, Garry et al., 2009). Therefore, it was hypothesised that factors involved in endometrial repair would be increased during menstruation. This chapter details endometrial IL-8, VEGF, AM, CTGF and ET-1 and their receptors in biopsies from across the menstrual cycle.

3.2 Methods

3.2.1 Human endometrial tissue collection

Endometrial tissue samples (n=41) were collected as described in section 2.1. Samples were categorised into menstrual, proliferative, early secretory, mid secretory and late secretory phase based on the woman's reported LMP, histological dating (Noyes et al., 1950) and serum oestradiol and progesterone levels taken at the time of biopsy. Endometrial samples assessed in this chapter are detailed in Table 5.

Table 5. Endometrial biopsies used for the studies presented in Chapter 3.
*progesterone values significantly less than those from the early- and mid-secretory phase ($p<0.001$, one way ANOVA with Tukey's multiple comparison post-test).

Stage of Cycle	Number of biopsies	Mean serum oestradiol levels, pmol/l (range)	Mean serum progesterone, nmol/l (range)
Menstrual	8	192.25 (55-514)	3.71* (1.24-10.59)
Proliferative	11	441.18 (79-1105)	2.81* (0.97-7.10)
Early Secretory	7	497.50 (289-841)	59.60 (23.2-112.91)
Mid Secretory	8	638.00 (242-1949)	64.30 (25.47-114.53)
Late Secretory	7	318.22 (59.09-819)	8.22* (1.06-16.95)

3.2.2 RNA extraction and Q-RT-PCR

RNA was extracted from all the endometrial samples and cDNA prepared and Q-RT-PCR performed as detailed in chapter 2.4. The expression of mRNA was measured for IL-8, CXCL2, VEGF, KDR, AM, CLR, RAMPs 1-3, CTGF, ET-1, and ET_B (Table 3, Chapter 2). A sample of liver cDNA was also prepared and included on each Taqman plate as a positive control. Housekeeping gene 18S showed no significant difference in tissue from across the menstrual cycle.

3.2.3 Immunohistochemistry

IL-8, VEGF, AM and CTGF proteins were localised in endometrial sections from across the menstrual cycle using the immunohistochemical protocols described in chapter 2.6. As AM, IL-8 and CTGF protein displayed marked changes across the menstrual cycle, the localisation and intensity of staining for these proteins was scored in the semi-quantitative manner detailed previously (Chapter 2.6.8).

3.2.4 *In vitro* tissue culture

If sufficient tissue was available, a portion of the endometrial biopsy from women at each stage of the menstrual cycle was collected in PBS (n=20), as described in Chapter 2.1. Tissue was weighed and incubated on raised platforms in 1ml serum free RPMI 1640 medium with penicillin (50 μg/ml, Sigma), streptomycin (50μg/ml, Sigma) and gentamycin (5μg/ml, Sigma) for 24h. The culture supernatant was collected and stored at -20°C before analysis by IL-8 ELISA (Chapter 2.7.1). Results were normalised to tissue weight.

3.2.5 Capillary tube formation assay

The angiogenic potential of human endometrial tissue and recombinant IL-8 was assessed using the capillary tube formation assay described in Chapter 2.8. HUVECs were treated with recombinant human IL-8 (R&D Systems, Oxford, UK) at a concentration of 0.5ng or 20ng in 250μl RPMI media. Alternatively, HUVECs were treated with the supernatants from weighed endometrial explants from the mid-secretory and menstrual phase cultured in RPMI for 24h. Each dose of IL-8 and each culture supernatant were assessed in triplicate in three separate experiments.

3.2.6 Statistical analysis

For mRNA and protein in endometrial biopsies from across the menstrual cycle, results were expressed as a quantity relative to a comparator, a sample of RNA from the liver. Significant differences in mRNA expression, protein levels and semi-quantitative immunohistochemical scoring were determined using Kruskal-Wallis non-parametric tests with Dunn's multiple comparison post-tests (GraphPad Prism Software, Inc., San Diego, CA). Samples that had a value more than two standard deviations greater or less than the mean for the group were considered outliers and removed from statistical analysis.

3.3 Results

3.3.1 Expression of IL-8 mRNA across the menstrual cycle

IL-8 mRNA was present at low levels in endometrium from the proliferative, early- and mid-secretory stages of the menstrual cycle (Figure 13). A non-significant increase in IL-8 mRNA was seen in the late secretory phase. By the menstrual stage, levels of IL-8 had risen significantly when compared to endometrium from the proliferative ($p<0.01$), early secretory ($p<0.001$) and mid secretory stage ($p<0.05$).

3.3.2 Immunolocalisation of IL-8 in the human endometrium

Immunolocalisation of IL-8 demonstrated positive cytoplasmic staining in glandular epithelial (GE), surface epithelial (SE), stromal cells (St), and perivascular cells in endometrium from the menstrual phase of the cycle (Figure 14 C, D). In contrast, proliferative phase endometrium displayed negligible staining for IL-8, even in perivascular cells (Figure 14 E, F). During the secretory phase GE cells were occasionally weakly stained for IL-8 (Figure 14 G, I, K). However, perivascular staining was maintained in the secretory phase (Figure 14 H, J, L).
Semi-quantitative scoring of IL-8 staining intensity revealed that the strongest staining was in the GE and perivascular cells (Figure 15 A, D). IL-8 perivascular staining was significantly increased during the menstrual phase of the cycle, when compared with the proliferative ($p<0.05$), early secretory ($p<0.01$) and mid secretory ($p<0.05$) stages (Figure 15 D). There was a non-significant increase in IL-8 staining in GE and stromal cells during the menstrual phase (Figure 15 B, C).

3.3.3 Secretion of IL-8 protein from human endometrial biopsies

The amount of IL-8 protein secreted from endometrial biopsies cultured *in vitro* for 24h followed a similar pattern to IL-8 mRNA expression (Figure 16). Endometrium from the menstrual stage secreted significantly higher levels of IL-8 protein than tissue from the early and mid secretory phases ($p<0.05$). There was no significant decrease in IL-8 secreted protein between menstrual and proliferative explant culture supernatant.

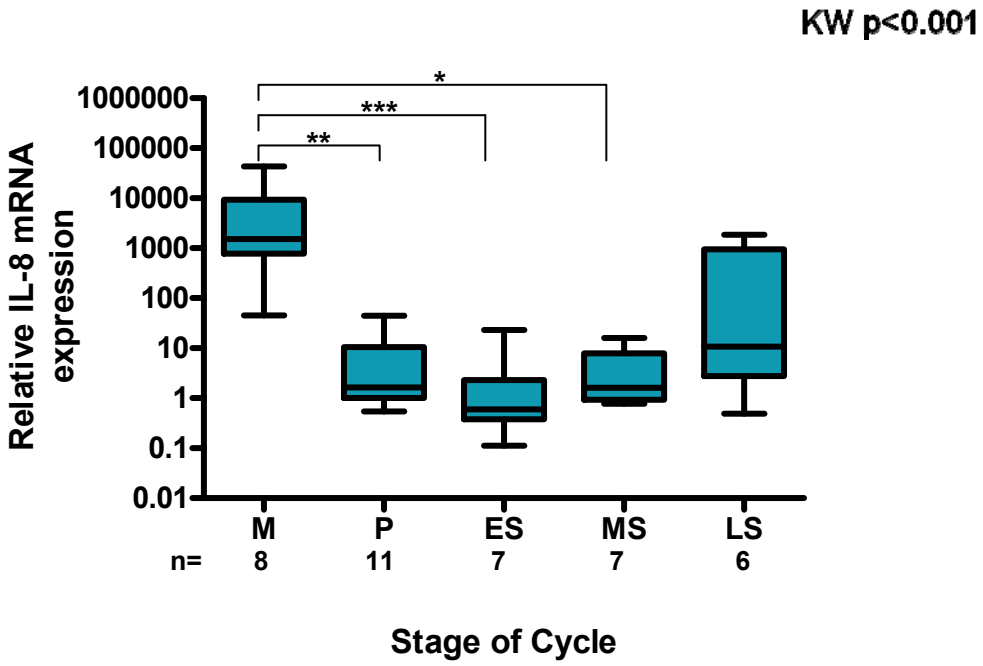


Figure 13. **IL-8 mRNA in endometrium from across the menstrual cycle.** IL-8 mRNA was quantified by Q-RT-PCR in endometrium from the menstrual (M), proliferative (P), early-secretory (ES), mid-secretory (MS) and late-secretory (LS) stages of the cycle. Note logarithmic scale on y-axis. (*p<0.05, **p<0.01, ***p<0.001, KW: Kruskal Wallis statistical test).

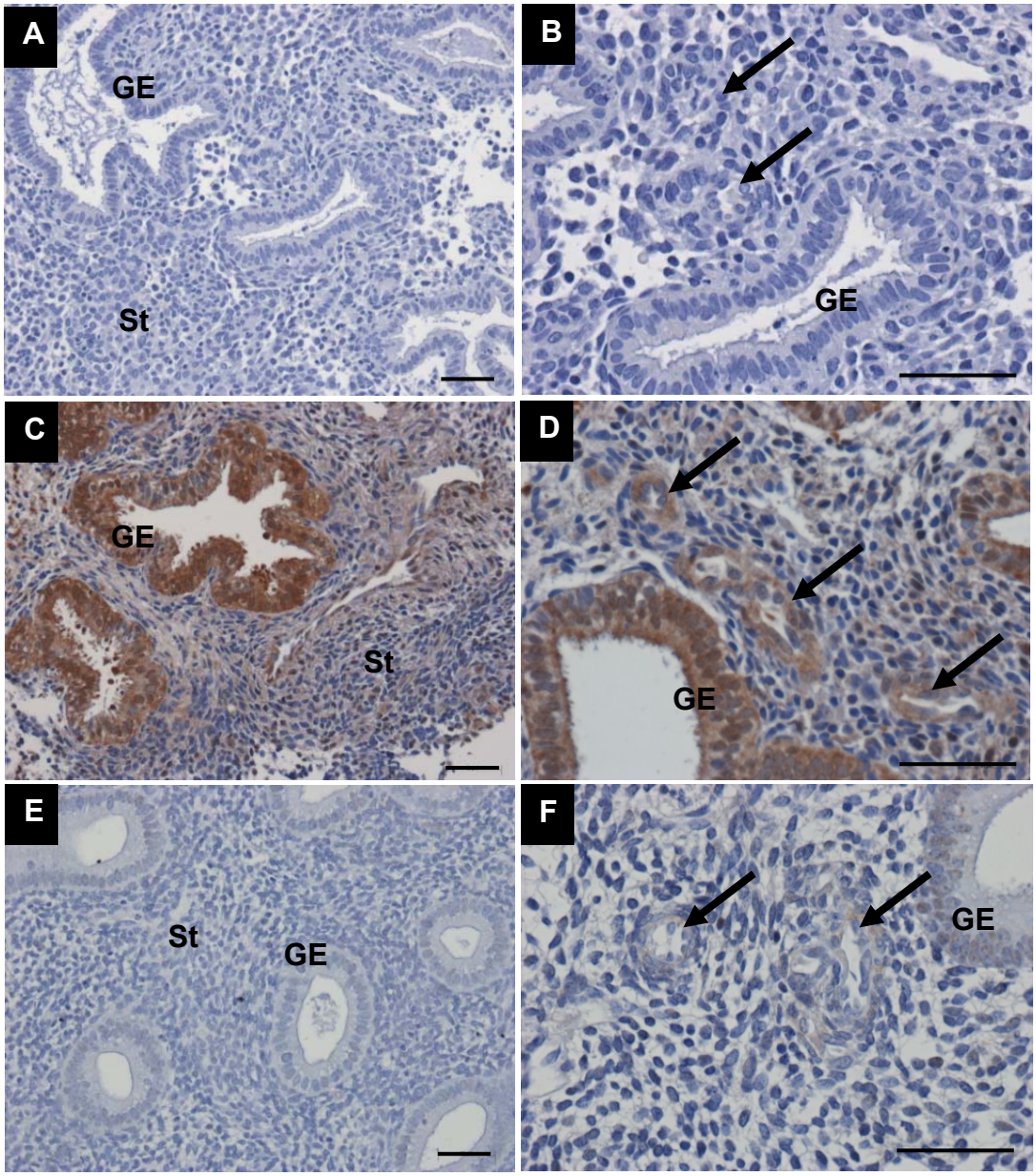
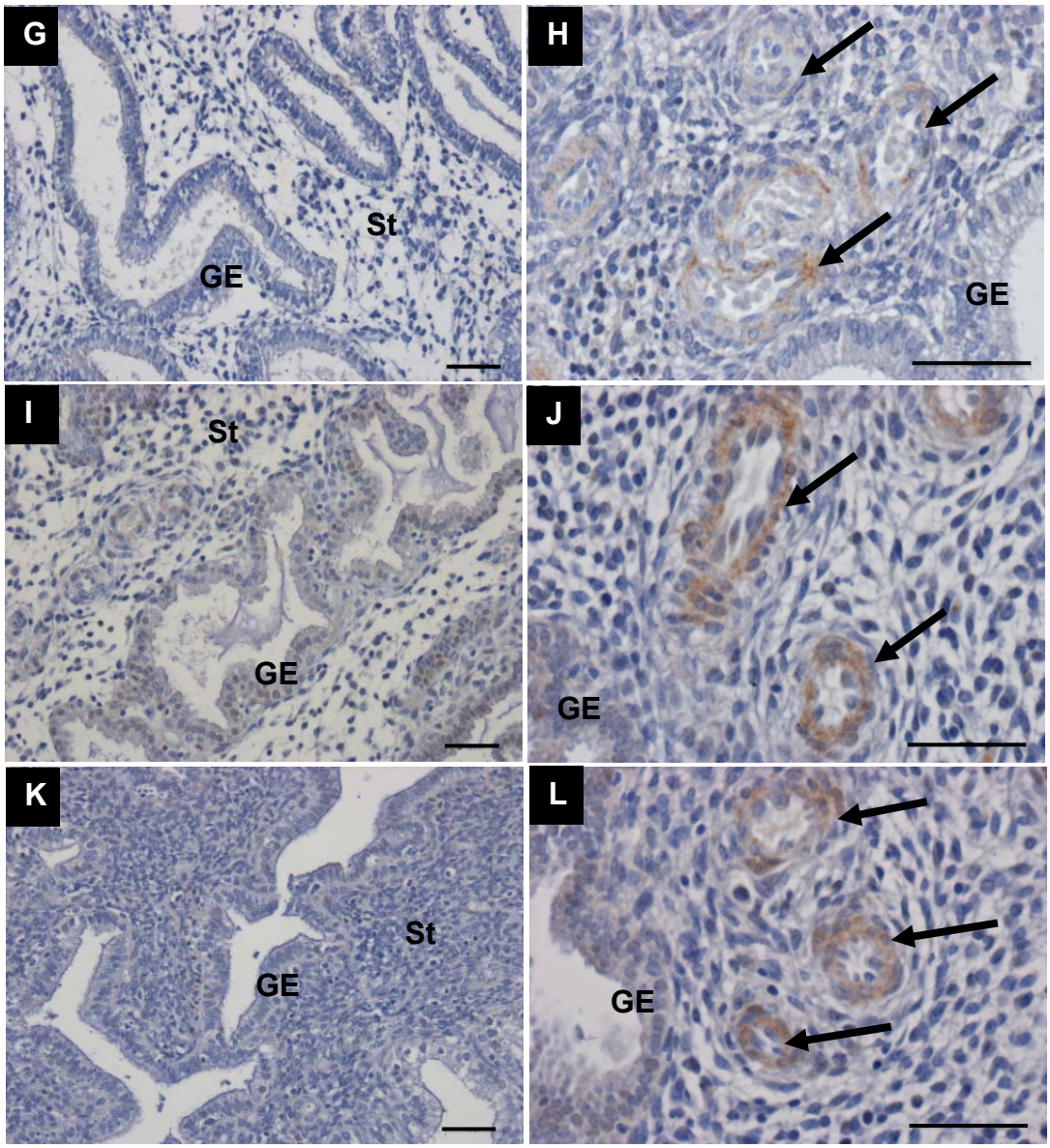


Figure 14. **Immunohistochemical staining for IL-8 in endometrium from across the menstrual cycle**. **(A, B)** menstrual endometrium stained with concentration matched IgG as negative controls **(C, D)** IL-8 stained menstrual endometrium, **(E, F)** proliferative phase endometrium, **(G, H)** early secretory endometrium, **(I, J)** mid-secretory endometrium, **(K, L)** late-secretory endometrium. Scale bar = 50μm. Arrows = perivascular cells. GE: glandular epithelial cells, St: stromal compartment. NB. Figure extends over two pages.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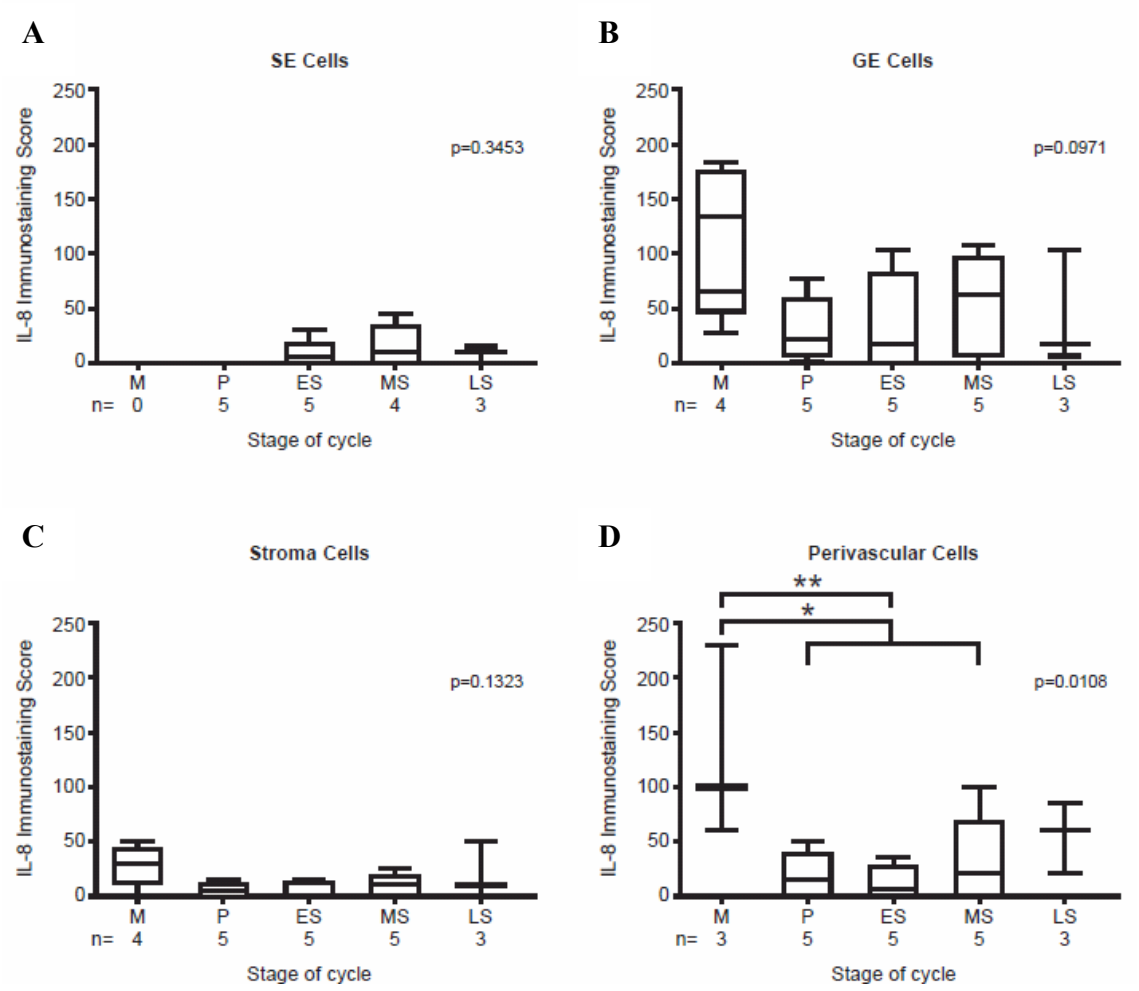


Figure 15. **Semi-quantitative scoring of IL-8 immunohistochemical staining in endometrium from across the menstrual cycle. (A)** endometrial surface epithelial cells, **(B)** glandular epithelial cells, **(C)** the stromal compartment, **(D)** perivascular cells. M**:** Menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. (* $p<0.05$, **$p<0.01$, KW Kruskal Wallis statistical test).

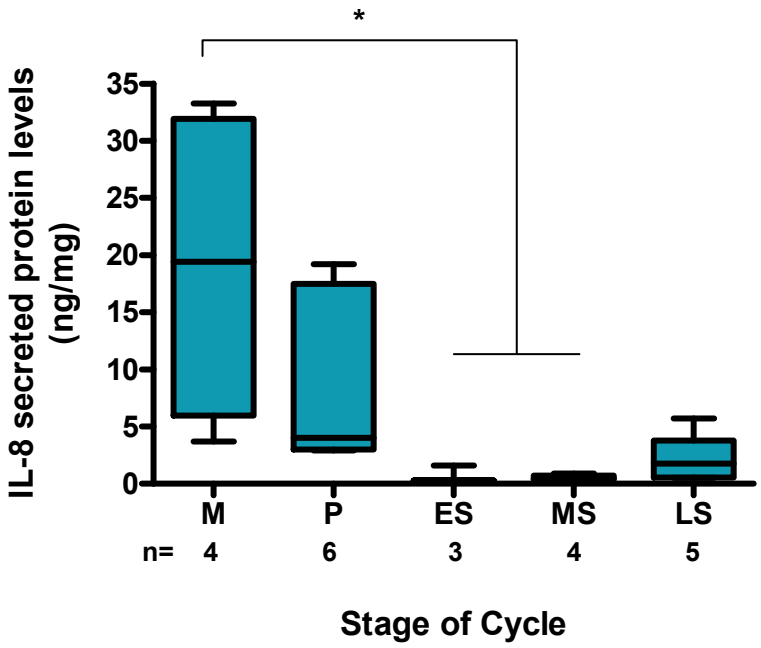


Figure 16. **Secreted IL-8 protein levels by endometrial explants from different stages of the menstrual cycle cultured *in vitro* for 24h.** M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. (*$p<0.05$).

3.3.4 Angiogenic potential of endometrial tissue and IL-8

To assess the angiogenic potential of endometrial IL-8 *in vitro,* human umbilical vascular endothelial cell (HUVEC) branching was quantified after various treatments. When treated with culture supernatants from menstrual tissue incubated for 24h *in vitro*, a significant increase in HUVEC capillary tube formation was observed when compared to cells treated with control (unconditioned) media (Figure 17A). No significant increase in tube formation was seen with supernatants from mid-secretory phase explants. These endometrial explants are likely to produce several angiogenic factors. To assess the contribution of IL-8 alone, HUVECs were also treated with recombinant human IL-8. The amount of IL-8 secreted by menstrual endometrial explants was 18.94 ± 7.57ng (mean ± SEM, median 19.4ng). Mid-secretory endometrium secreted the lowest levels of IL-8, 0.53 ± 0.13ng (mean ± SEM, median 0.44ng). Therefore, HUVECs were treated with control media, 20ng or 0.5ng of human recombinant IL-8. Treatment with 20ng IL-8 resulted in a significantly higher number of capillary tube branch points when compared with cells treated with 0.5ng IL-8 or control media (Figure 17B). Mid-secretory levels of IL-8 had no significant impact on branch points when compared to control media.

3.3.5 Expression of CXCL2 mRNA across the menstrual cycle

IL-8 receptor (CXCL2) mRNA was detected by Q-RT-PCR in human endometrial samples (Figure 18). There was significant variation in expression levels of CXCL2 across the menstrual cycle ($p<0.001$). Levels were maximal in menstrual endometrium, with significantly greater expression of CXCL2 mRNA than levels observed in proliferative ($p<0.01$) or early secretory ($p<0.001$) endometrial samples.

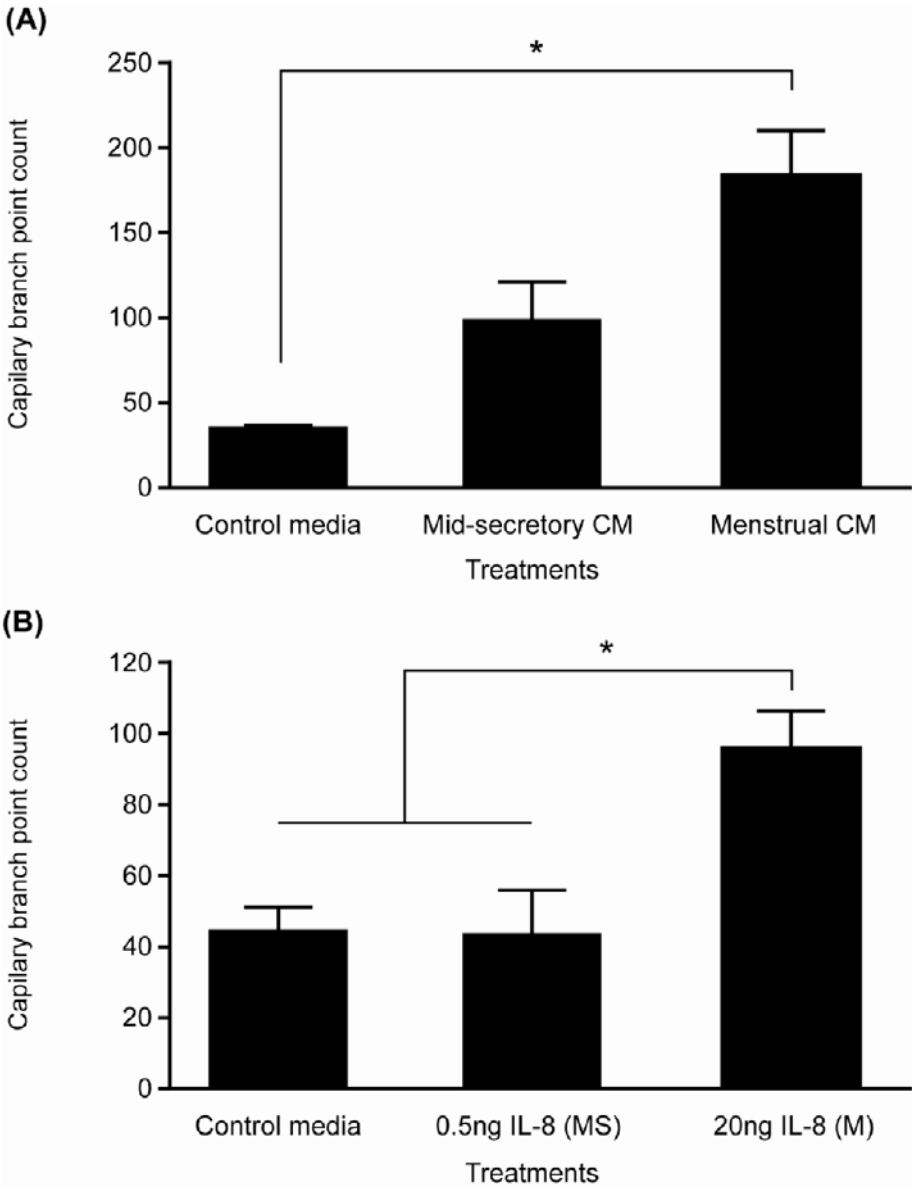


Figure 17. **Menstrual levels of IL-8 can up-regulate network formation in human umbilical vascular endothelial cells (HUVECs). (A**) The number of capillary tube branch points formed by HUVECs significantly increased when treated for 8h with conditioned media (CM) from menstrual endometrial biopsies cultured for 24h *in vitro* versus control media. No such increase was seen with CM from mid-secretory explants (n=4-5). **(B)** The number of capillary tube branch points formed by HUVECs treated with 20ng human recombinant IL-8 (M: menstrual levels) was significantly greater that those formed when treated with control media or 0.5ng IL8 (MS: mid secretory levels) (n=3). (*$p<0.05$).

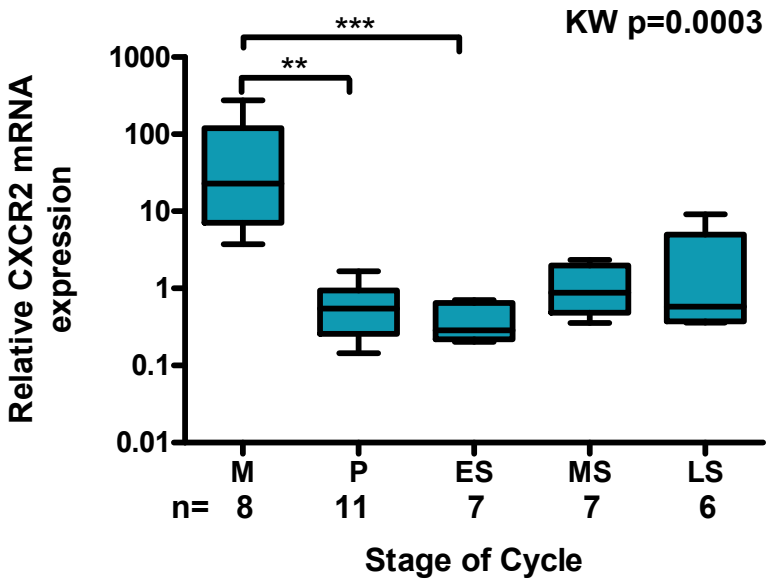


Figure 18. **CXCR2 mRNA measured by Q-RT-PCR in endometrium from across the menstrual cycle**. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. Note logarithmic scale on y-axis. (**p<0.01, ***p<0.001, KW: Kruskal Wallis statistical test).

3.3.6 VEGF mRNA across the menstrual cycle

VEGF mRNA was present at low levels in proliferative, early secretory and mid-secretory phase endometrial biopsies (Figure 19). There was a non-significant increase in VEGF mRNA expression during the late secretory phase. VEGF expression was maximal in menstrual phase endometrium, at levels significantly greater than those seen in proliferative and early secretory biopsies ($p<0.05$ and $p<0.001$ respectively).

3.3.7 VEGF secreted protein levels across the cycle

VEGF protein secreted by endometrial explants from across the menstrual cycle was measured by ELISA. VEGF protein secretion was significantly greater from proliferative endometrial explants compared with late secretory explants (Figure 20A). Immunolocalisation of VEGF protein in menstrual endometrium (Figure 20B) showed positive cytoplasmic staining in the surface epithelial cells (SE), glandular epithelial cells (GE), stromal compartment (St), perivascular cells and endothelial cells (arrows).

3.3.8 Expression of KDR mRNA across the menstrual cycle

VEGF receptor 2 (KDR) mRNA levels varied significantly across the menstrual cycle ($p<0.0001$) (Figure 21). KDR mRNA was greatest in late secretory and menstrual endometrial samples. Expression at these stages of the cycle was significantly greater that KDR mRNA levels found in proliferative and early secretory endometrium ($p<0.01$ and $p<0.001$ respectively).

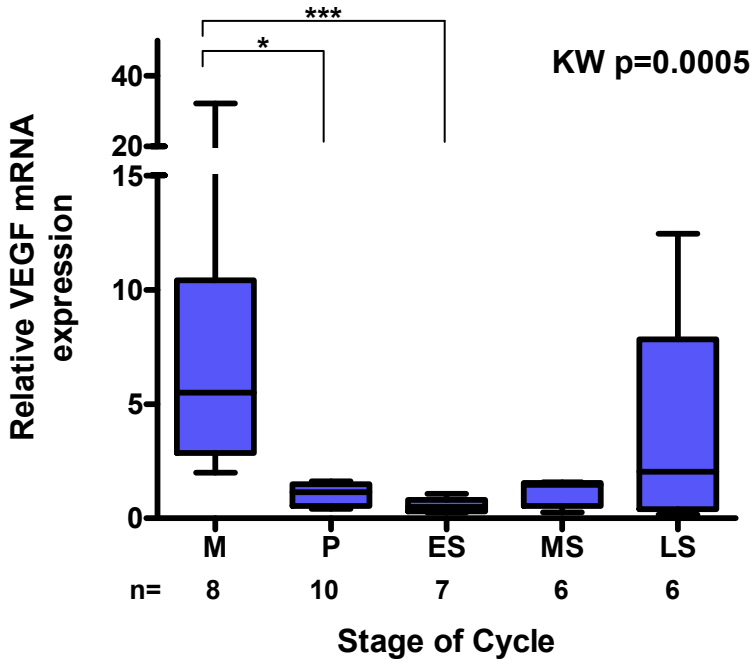


Figure 19. **Vascular endothelial growth factor (VEGF) mRNA in endometrium from across the menstrual cycle.** M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. (*p<0.05, ***p<0.001, KW: Kruskal Wallis statistical test)

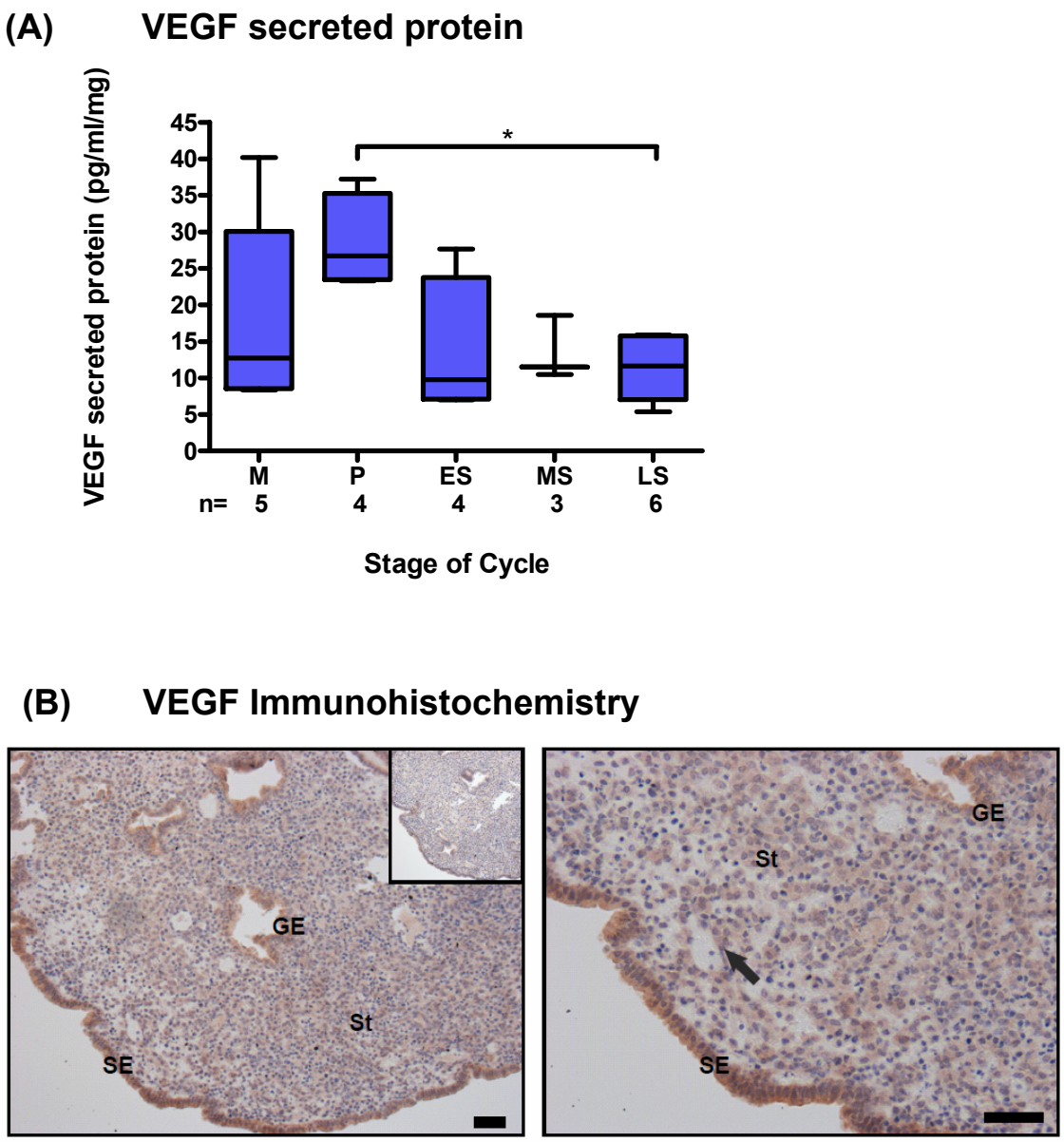


Figure 20. **Endometrial vascular endometrial growth factor (VEGF) protein levels across the menstrual cycle**. **(A)** Secreted VEGF protein levels from endometrial tissue explants at different stages of the menstrual cycle cultured *in vitro* for 24h. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. (*$p<0.05$). **(B)** Immunolocalisation of VEGF protein in menstrual endometrium. Scale bar = 50µm. Arrows = endothelial cells. SE: surface epithelial cells, GE: glandular epithelial cells, St: stromal compartment.

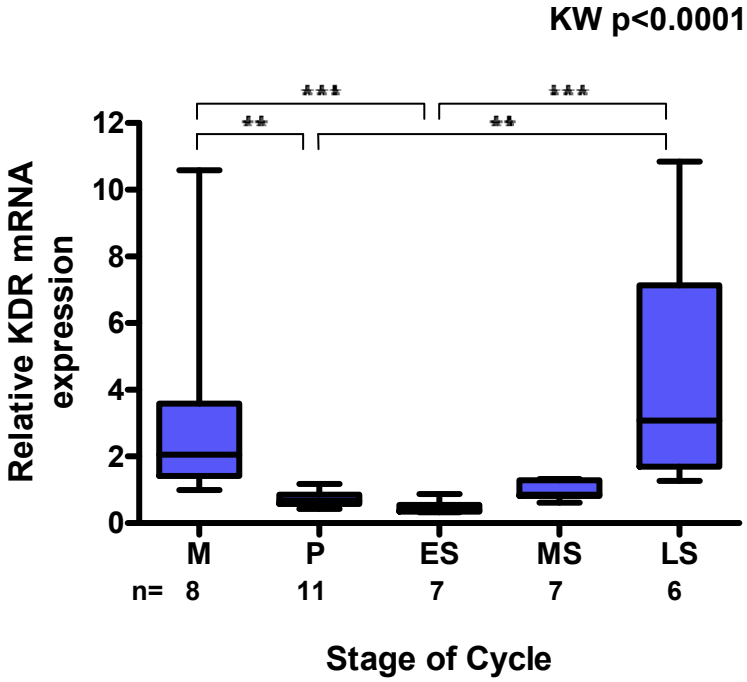


Figure 21. **Kinase insert domain receptor (KDR/VEGFR2) mRNA expression measured by Q-RT-PCR in endometrium from across the menstrual cycle**. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. (**p<0.01, ***p<0.001, KW: Kruskal Wallis statistical test).

3.3.9 AM mRNA across the menstrual cycle

AM mRNA expression was low during the proliferative and secretory phases of the menstrual cycle (Figure 22). Expression peaked at menstruation, when AM mRNA levels were significantly higher than during the proliferative phase ($p<0.001$).

3.3.10 Immunolocalisation of AM in the human endometrium

Immunostaining for AM was detected in a cytoplasmic location in the SE, GE, stromal compartment and endothelial cells of endometrium from the menstrual and proliferative phase (Figure 23A-D). Staining was less marked during the secretory phase (Figure 23E, F). Semi-quantitative assessment of staining revealed strongest staining in the SE cells, which did not vary significantly throughout the menstrual cycle (Figure 24E). In contrast, staining of the GE was maximal in the menstrual phase, being significantly increased compared to mid-secretory endometrium in both the functional and basal layers ($p<0.05$). There was also increased staining of proliferative phase GE cells but this did not reach statistical significance (Figure 24A,B). There was no significant difference in staining score of the stromal cell compartment across the cycle. However, highest levels of stromal cell staining were observed in menstrual and proliferative endometrial samples (Figure 24C,D). Endothelial cells from all phases stained positively for AM (Figure 24F).

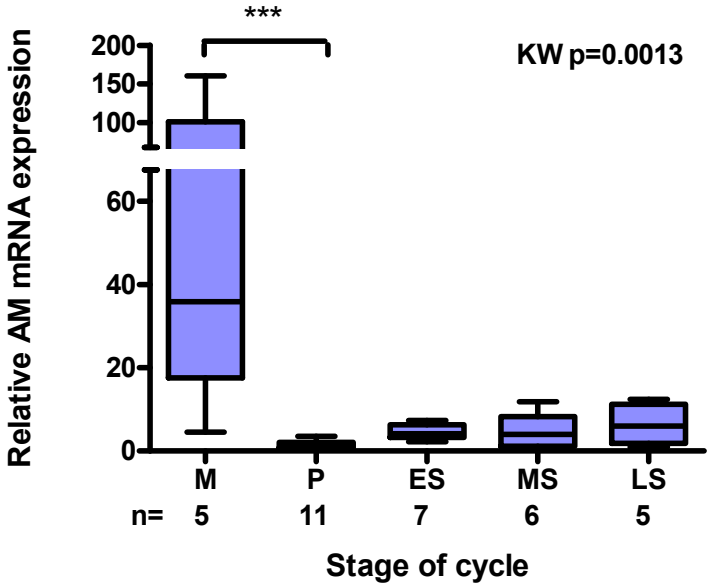


Figure 22. **Adrenomedullin (AM) mRNA measured by Q-RT-PCR in endometrium from across the menstrual cycle.** M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. (***p<0.001, KW: Kruskal Wallis statistical test)**.**

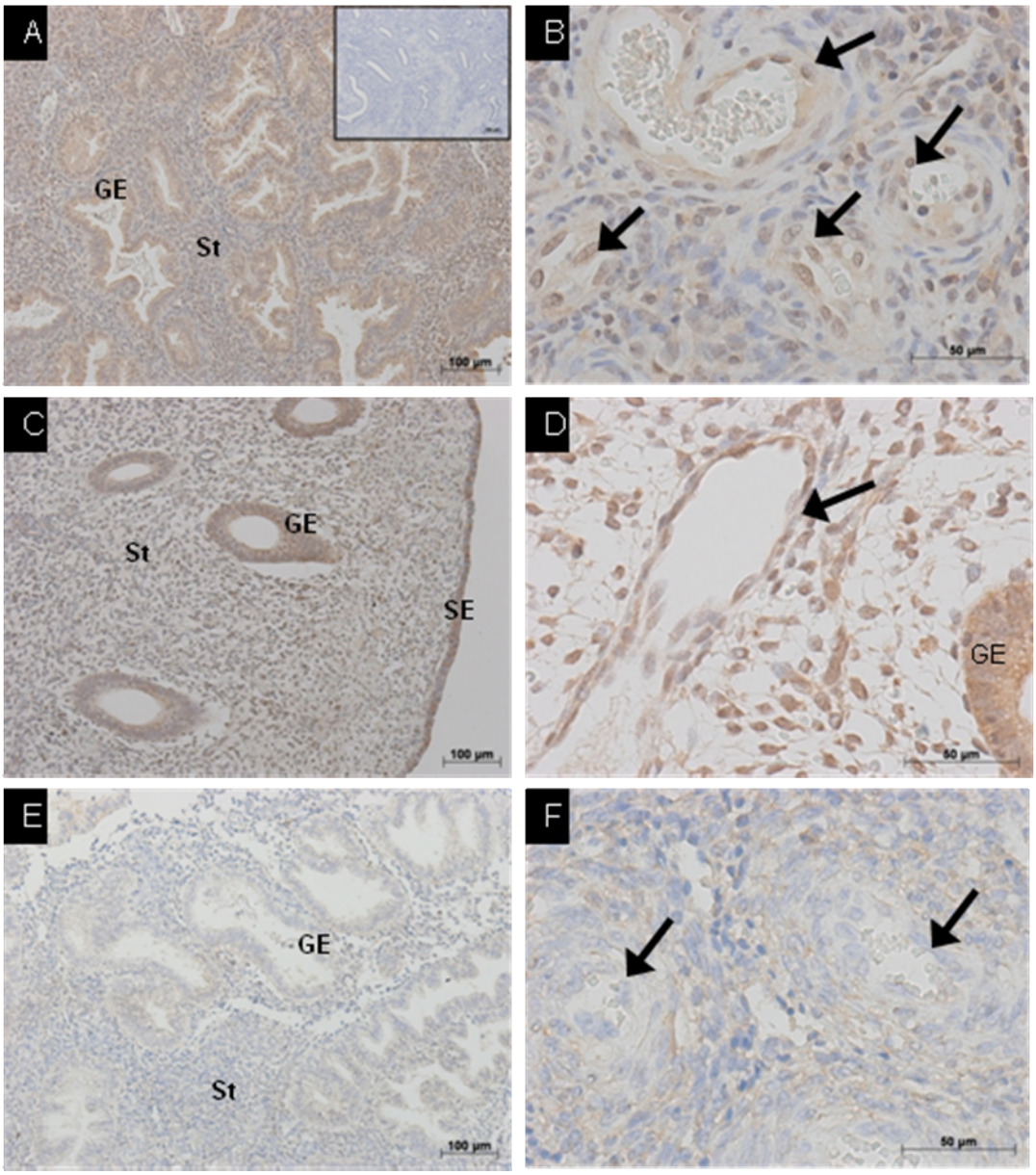


Figure 23. **Adrenomedullin immunohistochemical staining in endometrium from across the menstrual cycle**. **)** AM protein was present in the surface epithelium (SE), glandular epithelial cells (GE), the stromal compartment (St) and endothelial cells (arrows) during the menstrual **(A, B)** and proliferative **(C, D)** phase. Staining was markedly decreased during the secretory phase **(E, F)**. Insert = negative control. Scale bar 100µm or 50µm as marked.

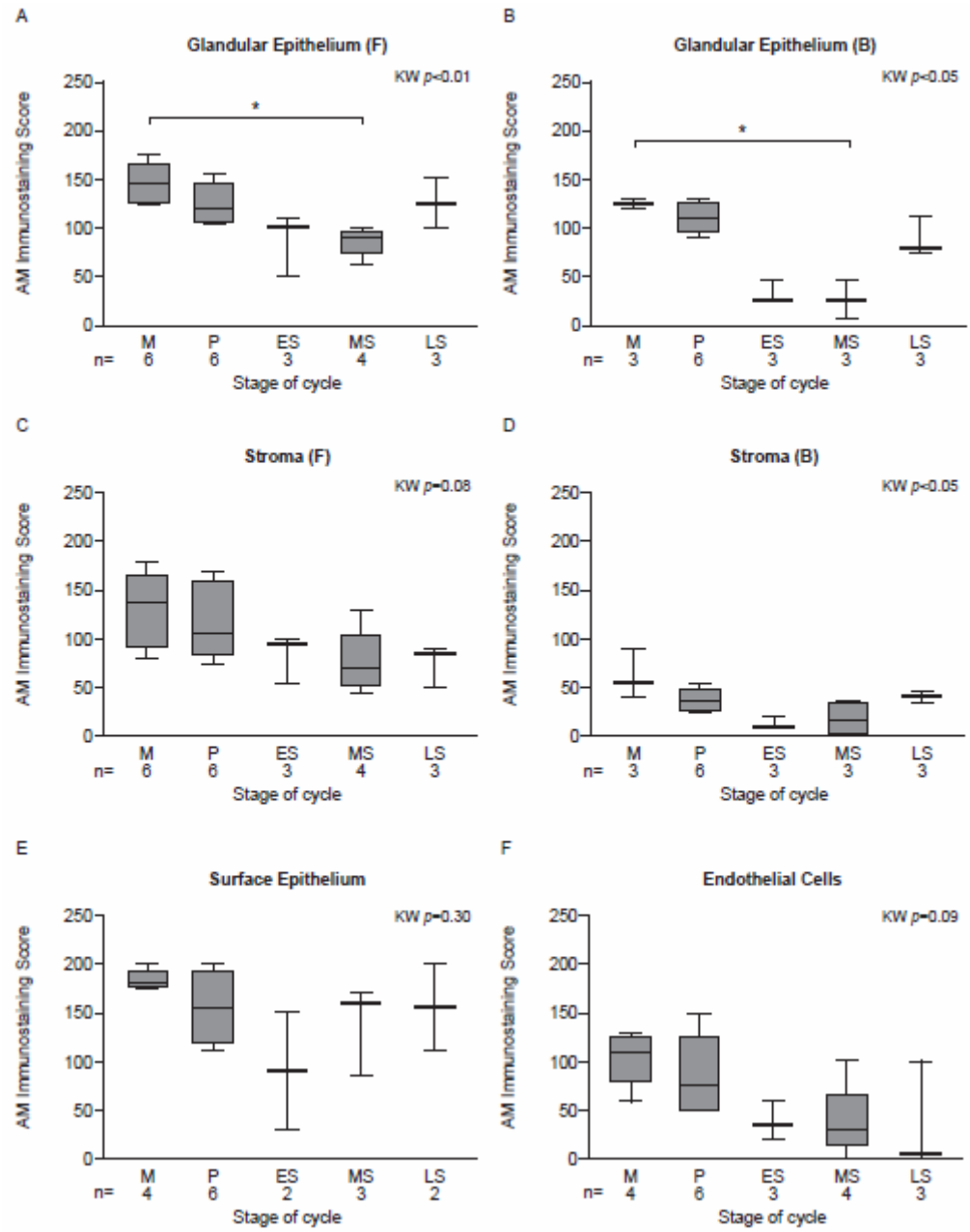


Figure 24. **Semi-quantitative scoring of adrenomedullin immunohistochemical staining**. (A) glandular epithelial cells of the functional (F) layer (B) glandular epithelial cells in the basal (B) layer (C) the stromal compartment of the functional endometrial layer (D) the stromal compartment in the basal layer (E) surface epithelial cells and (F) endothelial cells in both endometrial layers. M: menstrual, P: proliferative, ES: early-secretory, MS: mid-secretory, LS: late-secretory. KW: Kruskal Wallis statistical analysis. (*p<0.05).

3.3.11 CLR and RAMPs mRNA across the cycle

CLR mRNA was present throughout the menstrual cycle. Highest levels were observed in the mid-secretory phase, when CLR expression was significantly higher than in the proliferative ($p<0.01$) and late-secretory stage ($p<0.05$) (Figure 25A). The accessory protein RAMP1, which forms a heterodimer with CLR to preferentially bind calcitonin gene-related peptide, does not vary significantly across the menstrual cycle (Figure 25B). In contrast RAMP2, which binds to CLR to form the receptor for AM, is expressed in the human endometrium in a pattern similar to that of CLR (Figure 25C). RAMP2 mRNA expression was significantly higher in the early secretory phase than levels found in proliferative ($p<0.01$) or late secretory endometrium ($p<0.001$). Interestingly RAMP3, which also promotes preferential binding of CLR to AM, has the opposite expression pattern in the endometrium (Figure 25D). The highest levels of expression were seen during the menstrual and late secretory phases. Expression of RAMP3 in proliferative and early secretory endometrium was significantly decreased ($p<0.05$ and $p<0.001$ respectively) when compared to levels during the menstrual and late secretory phase.

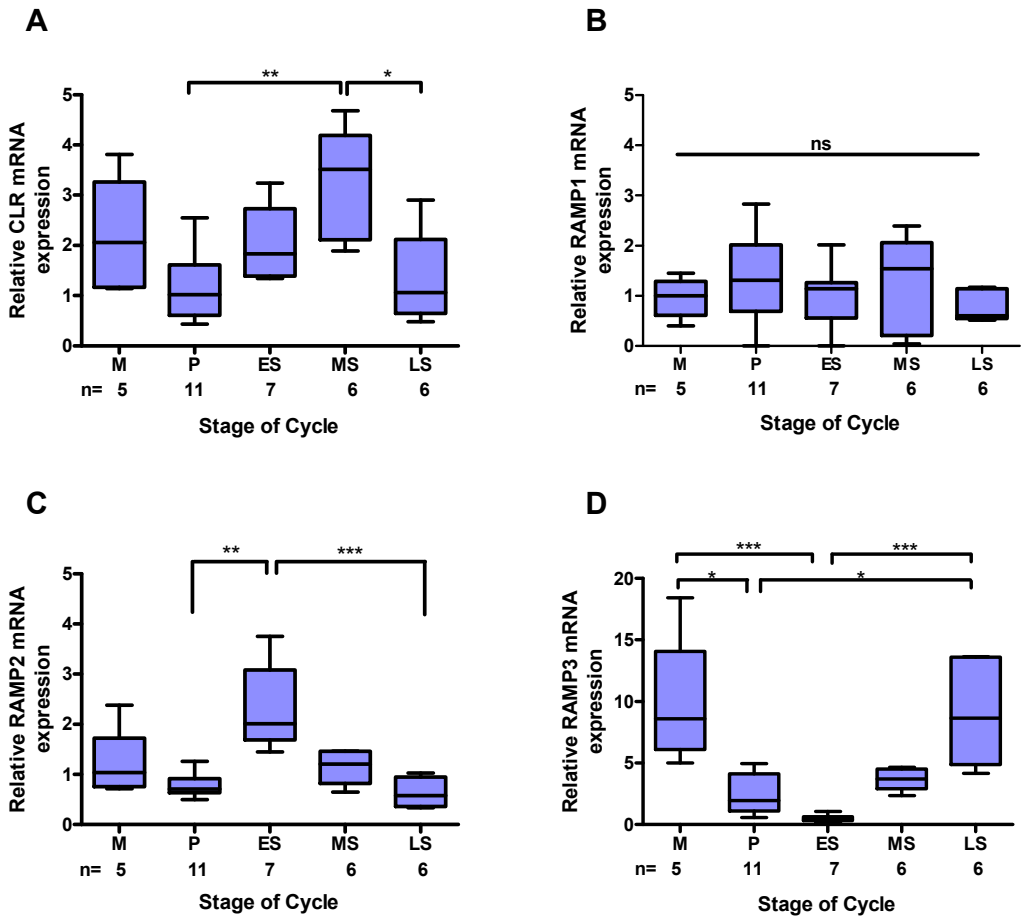


Figure 25. **Calcitonin receptor like receptor (CLR) and receptor activity modifying protein (RAMP) mRNA in human endometrium from across the menstrual cycle.** (A) CLR mRNA expression was highest in the mid-secretory phase (B) RAMP1 expression does not change significantly across the cycle (C) RAMP2 expression peaked during the early secretory phase (D) RAMP3 mRNA expression was greatest in late secretory and menstrual phase endometrium. M: menstrual, P: proliferative, ES: early-secretory, MS: mid-secretory, LS: late secretory. (ns: no significant difference, *p<0.05, **p<0.01, ***p<0.001).

3.3.12 CTGF mRNA across the menstrual cycle

CTGF mRNA was present in the endometrium and varied significantly across the cycle ($p<0.01$) (Figure 26). Low levels of CTGF mRNA were present in proliferative and secretory endometrium. Menstrual phase biopsies displayed significantly increased CTGF mRNA expression compared to early- ($p<0.01$), mid- ($p<0.05$) and late- ($p<0.05$) secretory endometrium.

3.3.13 Immunolocalisation of CTGF in endometrial biopsies

CTGF was immunolocalised to the cytoplasm of SE, GE, stromal and vascular endothelial cells at all stages of the menstrual cycle (Figure 27). There was an obvious reduction in staining intensity during the secretory phase (Figure 27E, F). Intense staining of occasional cells in the stromal compartment of proliferative endometrium was observed (Figure 27H). These cells were usually found in close proximity to the endometrial vasculature. Semi-quantitative scoring of immunohistochemical staining revealed significantly greater staining of the GE, SE and stromal cells in proliferative phase endometrium when compared to mid-secretory endometrium ($p<0.001$, $p<0.01$, $p<0.01$ respectively) (Figure 28).

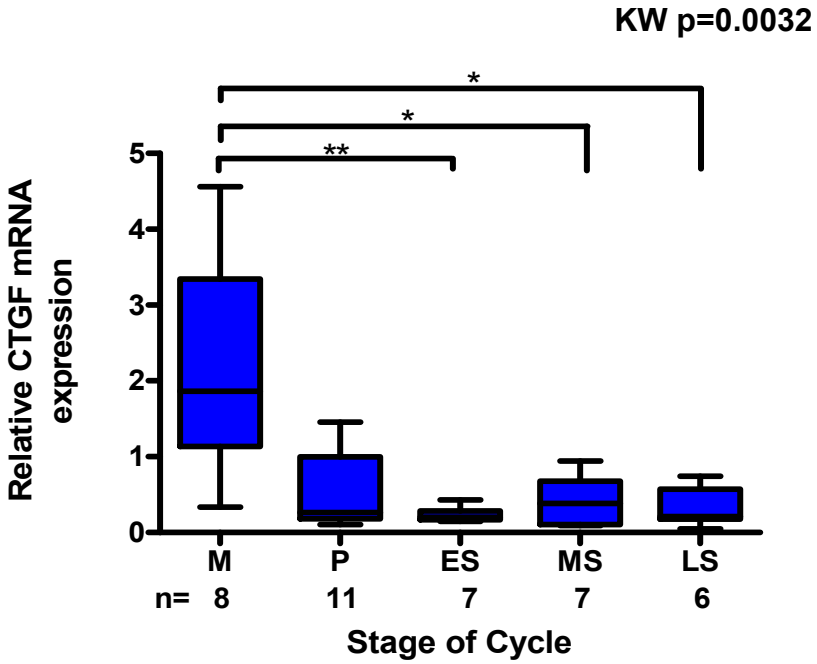
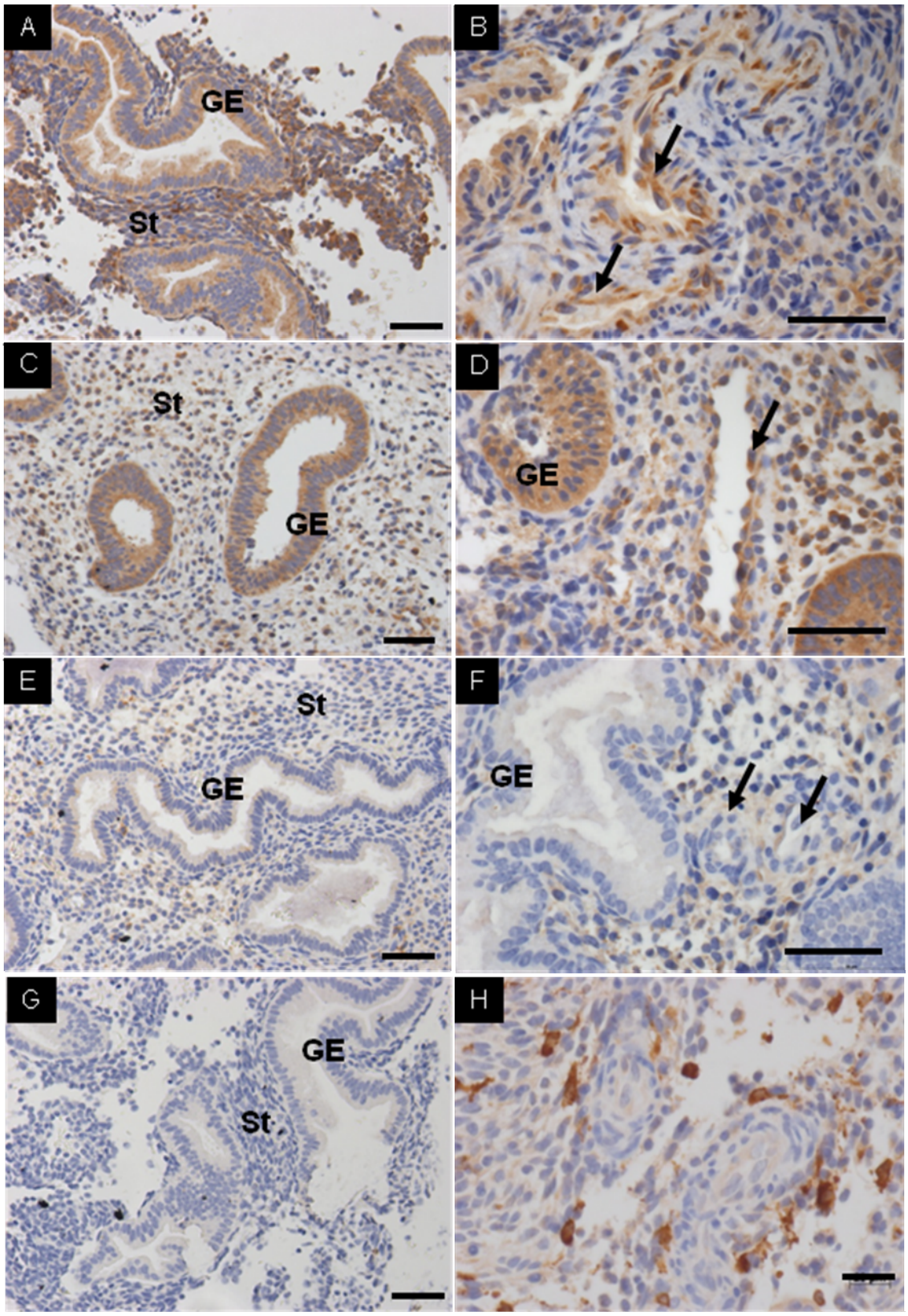


Figure 26. **Connective tissue growth factor (CTGF) mRNA in human endometrium from across the menstrual cycle.** M: menstrual, P: proliferative, ES: early-secretory, MS: mid-secretory, LS: late secretory. (*p<0.05, **p<0.01, KW: Kruskal Wallis statistical test).

Figure 27. **Connective tissue growth factor immunohistochemical staining in endometrium from across the menstrual cycle**. (A, B) menstrual phase endometrium, (C, D) proliferative phase endometrium, (E, F) mid-secretory phase endometrium, (G) negative control menstrual endometrium incubated with the primary antibody pre-absorbed with blocking peptide, (H) intense staining of occasional cells in the stromal cell compartment of proliferative endometrium. Scale bar = 50μm. Arrows = endothelial cells. SE: surface epithelial cells, GE: glandular epithelial cells, St: stromal compartment.


A
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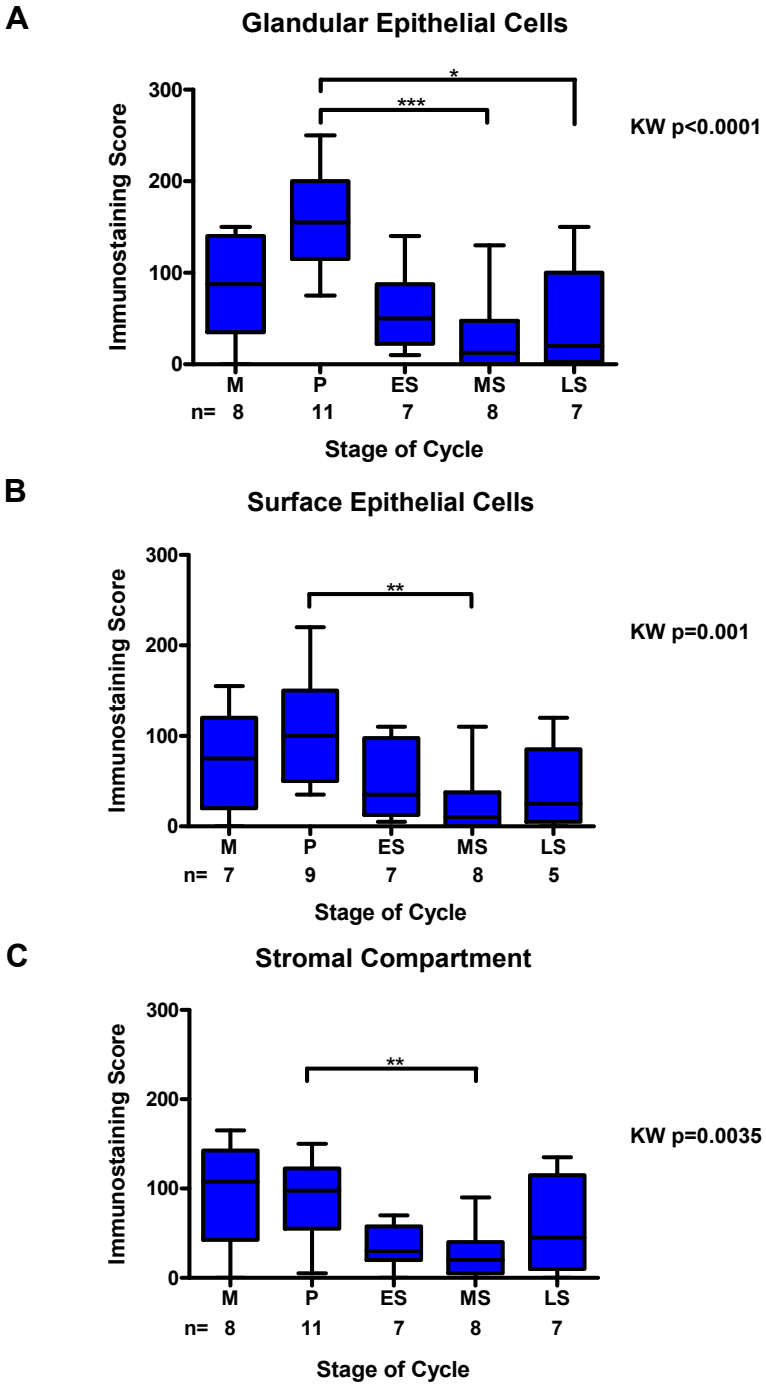


Figure 28. **Semi-quantitative scoring of endometrial connective tissue growth factor immunohistochemical staining across the menstrual cycle.** (A) Glandular epithelial cells, (B) surface epithelial cells, (C) the stromal cell compartment. M: menstrual, P: proliferative, ES: early-secretory, MS: mid-secretory, LS: late secretory. (*p<0.05, **p<0.01, ***p<0.001).

3.3.14 ET-1 mRNA across the menstrual cycle

ET-1 mRNA levels are significantly increased in menstrual endometrium relative to proliferative ($p<0.001$) and early-secretory ($p<0.001$) samples. In addition, there is a non-significant increase in ET-1 mRNA expression in mid- and late-secretory endometrial samples (Figure 29).

3.3.15 Expression of ET_B mRNA across the cycle

ET_B mRNA levels changed significantly when quantified in endometrial samples from across the menstrual cycle ($p<0.001$) (Figure 30). Maximal expression was observed in menstrual and late-secretory endometrial samples. Low levels of ET_B expression were observed in proliferative and early secretory endometrium, with increasing levels present in the mid-secretory phase. Menstrual and late secretory ET_B mRNA levels were significantly greater than those present in proliferative and early-secretory samples ($p<0.05$ and $p<0.001$ respectively).

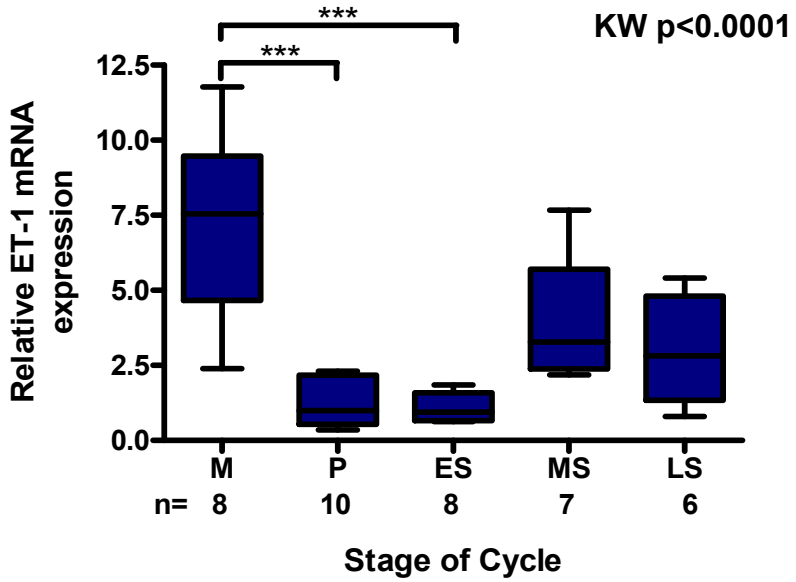


Figure 29. **Endothelin-1 (ET-1) mRNA in human endometrium from across the menstrual cycle**. M: menstrual, P: proliferative, ES: early-secretory, MS: mid-secretory, LS: late secretory. (***p<0.001, KW: Kruskal Wallis statistical test).

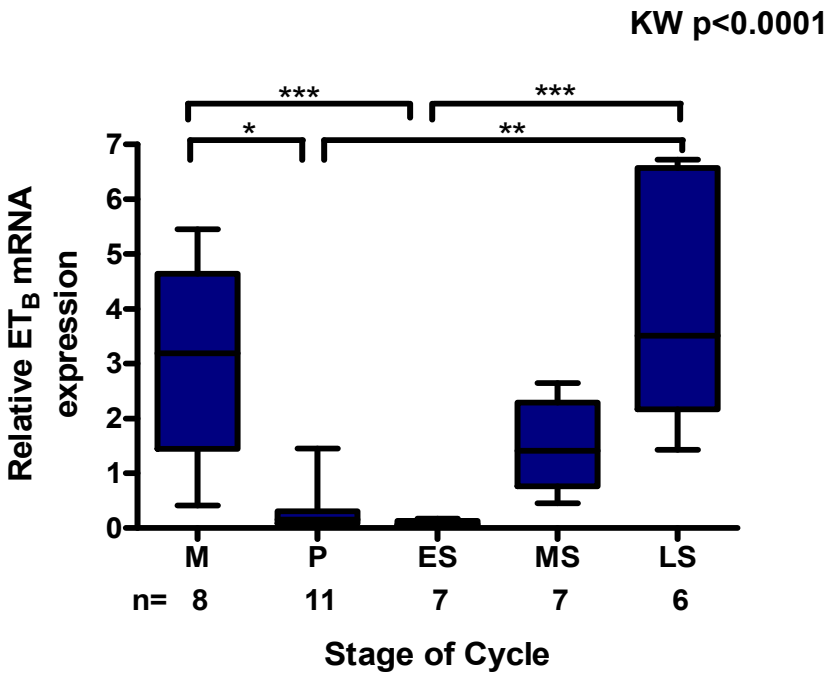


Figure 30. **Endothelin receptor B (ET_B) mRNA in human endometrium from across the menstrual cycle.** M: menstrual, P: proliferative, ES: early-secretory, MS: mid-secretory, LS: late secretory. (*p<0.05, **p<0.01, ***p<0.001, KW: Kruskal Wallis statistical test).

3.4 Discussion

The results presented in this chapter describe the significant up-regulation of putative endometrial repair factors during the menstrual phase of the cycle. IL-8, VEGF, AM, CTGF and ET-1 mRNAs are all maximal during menstruation. In addition, protein levels of these factors are highest during the menstrual and/or proliferative phase. Furthermore, endometrial mRNA was detected for the receptors CXCL2, KDR, CLR and RAMPs 1-3, and ET_B. Endometrial repair has been shown to commence on day two of the cycle (Ludwig and Spornitz, 1991). Hence, these findings of maximal repair factor expression during menstruation are consistent with a role in endometrial repair. Detection of endometrial expression of their receptors lends further support to an active role in the repair process.

The data presented in this chapter were generated from examination of endometrial biopsies collected with an endometrial sampler (Pipelle ®). The majority of endometrium collected with this method is from the functional layer. As this layer is sloughed off during menses, it was traditionally believed that endometrial repair was governed by factors present in the basal layer. However, a recent study of menstrual endometrium revealed an increase in genes associated with extracellular matrix biosynthesis in stromal cells from the functional layer when compared to those from the basal layer (Gaide Chevronnay et al., 2009). Over expression of these genes, which included IL-8 (>4 fold increase), suggests that fragments of the functional layer of endometrium make an active contribution to the endometrial repair process. In addition, endometrial tissue used in this study was collected from women with a subjective complaint of HMB. Previous studies have shown that 64% of women with subjective heavy loss actually had an objective measurement of <80ml (normal menstrual loss) (Wyatt et al., 2001). Collection of tissue from women without this subjective complaint is difficult as they rarely attend gynaecological services. However, reproduction of these findings in women with objectively measured NMB would further support the role of these factors in endometrial repair.

3.4.1 Interleukin-8

The significant elevation of IL-8 during menstruation described herein is in agreement with the findings of Jones *et al.,* who reported negligible levels of IL-8 mRNA at all stages of the cycle with the exception of a dramatic up-regulation at menstruation (Jones et al., 2004). This study extends these findings by also demonstrating significant elevations in menstrual IL-8 protein. Other studies have found an increase in endometrial IL-8 mRNA and protein during the late secretory phase of the menstrual cycle (Arici et al., 1998, Milne et al., 1999). Importantly, these studies did not examine tissue from the menstrual phase; therefore any increase in IL-8 during this stage was undetected. Consistent with findings detailed herein, endometrial IL-8 has been previously localised to the perivascular cells by *in situ* hybridisation (Milne et al., 1999). The exact nature of the cellular component in the perivascular region is still to be determined. Perivascular IL-8 production may be from macrophages, vascular smooth muscle cells, fibroblasts or myofibroblasts. Negative staining in endothelial cells means they are less likely to play a major role in perivascular IL-8 production. Further studies are required to determine which cell types are involved. The bioavailability of IL-8 in the endometrium appears to be regulated by aminopeptidase N (Seli et al., 2001). This IL-8 inactivating enzyme is produced by endometrial stromal cells, is lowest during the early proliferative phase and appears to be increased by oestrogen. This pattern and regulation ensures active IL-8 is present in the perimenstrual endometrium.

IL-8 exerts its effects by binding to chemokine receptors CXCR1 and CXCR2. Described herein is the presence of endometrial CXCL2 mRNA expression, with significant up-regulation in menstrual phase biopsies. CXCR1 and CXCR2 have previously been immunolocalised in the human endometrium and were present in epithelial and stromal cells (Mulayim et al., 2003). When intensity of staining was examined by these authors, CXCR1 staining was found to be significantly higher in the mid-secretory versus the proliferative phase in both stromal and epithelial cells. No variation in CXCR2 staining was detected across the cycle. Mulayim *et al.* did not examine menstrual phase endometrial samples. Menstrual samples may have

significantly elevated CXCR2 protein levels, reflecting the raised mRNA expression observed herein.

3.4.2 Vascular endothelial growth factor

This chapter also details a significant increase in VEGF mRNA in late-secretory and menstrual phase biopsies. Intense immunostaining of VEGF was observed in menstrual and proliferative phase endometrium, particularly in GE and endothelial cells. This discrepancy in maximal mRNA and protein levels, with an increase in protein subsequent to mRNA, may be indicitative of a delay between increased transcription/mRNA stabilisation and protein translation. Maximal levels in the perimenstrual phase is consistent with findings in the rhesus macaque endometrium, where heightened expression of VEGF was present in the newly formed surface epithelium and stroma during post-menstrual repair (Nayak and Brenner, 2002). In addition, a limited number of human endometrial immunohistochemical and *in situ* hybridisation studies have also shown increased VEGF expression in the menstrual phase in the glandular epithelial cells (Zhang et al., 1998, Charnock-Jones et al., 1993). Lack of menstrual tissue availability has resulted in many studies not examining VEGF at this stage of the cycle (Gargett et al., 1999, Li et al., 1994). Delineation of endometrial VEGF during menstruation is essential to determine its contribution to early endometrial repair. VEGF is a potent angiogenic factor that stimulates endothelial cell proliferation and migration, increases vascular permeability and stimulates the expression of tissue factor, an initiator of coagulation, (Ferrara, 2004, Blum et al., 2001, Fan et al., 2008). Therefore, it has an attractive role in the repair of damaged blood vessels at menstruation. A recent study of the decidualised mouse uterus and rhesus macaque endometrium, found that VEGF blockade with VEGF Trap completely inhibited neovascularisation and reepithelialisation during endometrial repair (Fan et al., 2008).

VEGF acts through two transmembrane tyrosine kinase receptors, VEGF receptor 1 (VEGFR1/Flt1) and VEGF receptor 2 (VEGFR2/Flk1/KDR), and both have been identified in the human endometrium (Nayak et al., 2000, Punyadeera et al., 2006). As most of the biological effects of VEGF appear to be mediated through KDR (Li et

al., 2002), the presence of this receptor was examined in endometrial samples from across the menstrual cycle. KDR mRNA correlated with VEGF expression in the endometrium, with highest levels expressed during the late-secretory and menstrual phase. This correlation of VEGF ligand and receptor has been described previously by immunohistochemistry in the rhesus macaque and human endometrium (Wei et al., 2004, Moller et al., 2001). A perimenstrual increase in KDR has also been previously demonstrated in the ovariectomised macaque model and in the human endometrium (Nayak et al., 2000). This increase in KDR was shown to be limited to the functional endometrial layer and was not observed for VEGF receptor type 1. Co-ordinated expression of pro-angiogenic VEGF and its receptor in the human endometrium during the perimenstrual phase is consistent with a major contribution to the repair process.

3.4.3 Adrenomedullin

The data in this chapter also reveal a significant increase in AM mRNA during menstruation. In addition, protein levels were highest during the menstrual and proliferative stages; a temporal pattern consistent with a role in repair. AM has been detected previously in the human endometrium (Nikitenko et al., 2001, Laoag-Fernandez et al., 2000, Michishita et al., 1999). However, its expression at well defined stages of the menstrual cycle has not been characterised. These results demonstrate that AM mRNA and protein are maximal during the menstrual phase of the cycle. Immunolocalisation revealed AM in the SE, GE, stromal compartment and endothelial cells, in agreement with previous in-situ hybridization studies (Nikitenko et al., 2001).

Nikitenko *et al.* showed by RT-PCR that AM mRNA expression was present in the endometrium at lower levels than in the placenta (Nikitenko et al., 2000). However, the variation in AM mRNA levels at different stages of the cycle was not examined. At the protein level, Laoag-Fernandez *et al.* used immunohistochemistry to detect AM in endometrium from across the menstrual cycle (Laoag-Fernandez et al., 2000). They did not include menstrual endometrium in their study, presumably due to difficulties obtaining this tissue. In contrast to our findings, their study revealed little

positive staining for AM in early-mid proliferative endometrium, with more abundant staining in the secretory phase. These conflicting findings may be explained by a number of factors, including classification of tissue and antibody specificity. Most importantly, the endometrial samples used by Laoag-Fernandez *et al.* were from women undergoing hysterectomy for conditions such as leiomyomata, ovarian cancer, adenomyosis and cervical intraepithelial neoplasia. Neoplasia and fibroid changes have previously been shown to affect endometrial AM (Nunobiki et al., 2009, Hague et al., 2000). Therefore, results from this study may not be applicable to the normal endometrium.

The findings detailed herein, of positive AM staining in endometrium from the proliferative phase, are consistent with findings from a DNA microarray study of the mouse uterus (Watanabe et al., 2006). A dose-dependent increase in AM was observed with oestrogen treatment (correlation coefficient >0.95). In addition, this study identified that the oestrogen receptor (ER) binds to the promoter region of the AM gene, activating its transcription. These findings suggest that AM in the proliferative endometrium would be expected, as oestrogen is the dominant hormone in this phase. However, our study also revealed high levels of AM protein during the menstrual phase, when E_2 levels are low. This suggests an additional, E_2 independent, mechanism of AM up-regulation (see Chapter 4.3.2).

The data in this chapter also demonstrate differential expression of CLR and RAMPs in the human endometrium at different stages of the menstrual cycle. Nikitenko *et al.* previously identified the endometrial expression of CLR, RAMP1, RAMP2 and RAMP3 by RT-PCR but did not assess their levels across the cycle (Nikitenko et al., 2001). The observation of high CLR mRNA during the early-secretory phase, with low levels seen during the menstrual and proliferative phases, is inversely correlated to the pattern of endometrial AM expression. Internalisation and desensitisation of the endogenous CLR was observed in endothelial cells on exposure to AM (Nikitenko et al., 2006a), suggesting the function of CLR is tightly regulated by its ligand. A similar pattern of reduced CLR expression was seen in the mouse model of sepsis, where AM expression is markedly up-regulated (Ono et al., 2000).

Interestingly, the accessory protein that allows preferential binding of CGRP (RAMP1) does not change significantly across the cycle. However, RAMP2 and RAMP3, which allow AM binding when heterodimerised with CLR, show marked changes. RAMP2 has low expression during the menstrual and proliferative phases, when AM levels are high. In contrast, RAMP3 mRNA shows a similar pattern to AM. This switch from RAMP2 to RAMP3 expression as AM mRNA increases has been previously shown in human coronary artery smooth muscle cells and in the mouse lung (Ono et al., 2000, Nagoshi et al., 2004) but it is believed that this is the first report of this pattern in human endometrial tissue. Further study of this receptor and its binding proteins is required to confirm their functional role within the endometrium. These accessory proteins may affect AM responsiveness, with RAMP2 and RAMP3 having differential effects. The finding that their mRNA expression varies significantly across the menstrual cycle suggests hormonal responsiveness and an active role in endometrial physiology.

3.4.4 Connective Tissue Growth Factor

CTGF is a member of the CCN family of growth regulators and has a putative role in endometrial repair. Data in this chapter demonstrate maximal CTGF mRNA in menstrual phase endometrium. CTGF protein showed intensely positive immunohistochemical staining during the proliferative phase, subsequent to maximal mRNA expression. CTGF protein was significantly elevated in GE, SE and stromal cells when compared to the mid-secretory phase. This is the first description of CTGF mRNA and protein staining intensity in human endometrium from all stages of the menstrual cycle. Previous studies have immunolocalised CTGF in the human endometrium and found similar staining of GE, SE, stromal and endothelial cells but did not examine endometrium from the menstrual phase (Uzumcu et al., 2000). Porcine CTGF also demonstrates clear endometrial changes during oestrous. CTGF was detected by Northern blot in pig uterine luminal flushings on days 10 to 16 and during early pregnancy (Ball et al., 1998). Interestingly, intense staining of occasional stromal compartment cells occurred during the proliferative phase. These may be immune cells. Co-localisation of CTGF and inflammatory cell markers by immunohistochemistry may further delineate the nature of these cells and the role of

CTGF in the proliferative endometrium. Other members of the CCN family also show significant variation in human endometrial expression across the cycle. The pro-angiogenic factor cysteine rich protein 61 (CYR61) was found to be significantly elevated during menses (Gashaw et al., 2008) and authors proposed it contributes to repair, growth and maturation of the endometrial vasculature after menstruation.

The mechanism by which CTGF exerts its effects remains undefined at a molecular level; therefore examination of receptors for this factor was not possible. CYR61 has been shown to bind to integrin $\alpha_v\beta_3$ or integrin $\alpha_{IIb}\beta_3$ to mediate cell adhesion and migration (Kireeva et al., 1998, Jedsadayanmata et al., 1999) and CTGF may elicit its biological effects in a similar manner. Alternatively, it has been proposed that the CCN family may not signal exclusively via cell surface receptors but rather are a component of the ECM and bridge the functional gap between structural and active molecules (Lau and Lam, 1999). Other components of the ECM are known to have a fundamental role in the repair process and have a similar endometrial pattern of expression to that of CTGF. Fibronectin (FN) is a large glycoprotein that interacts with specific integrins to enhance cell adhesion and migration during wound repair (Kim et al., 1992). Utilizing the ovariectomised rhesus macaque model, Cao et al. demonstrated a 50 fold increase in FN from the secretory to the menstrual phase alongside a 17 fold increase in its receptor, Integrin-β1 (Cao et al., 2007). Real-time PCR, in situ hybridization and immunohistochemistry confirmed these findings and localized the increase in FN expression to the uppermost endometrial glands and luminal epithelium. Significant increases in the adhesive molecules CTGF and FN in the functional layer of the endometrium at this time suggest an involvement in surface healing post-menstruation.

3.4.5 Endothelin-1

ET-1 is best known for its actions as a potent vasoconstrictor but it also has angiogenic and mitogenic properties, reviewed in (Simonson and Dunn, 1990). Data presented in this chapter illustrate a significant up-regulation of ET-1 mRNA expression in the menstrual endometrium. This finding is in agreement with previous studies which found significantly greater ET-1 mRNA in endometrium from the

perimenstrual phase (Economos et al., 1992, Kubota et al., 1995). Comprehensive assessment of human endometrial ET-1 protein levels by immunohistochemistry and radioimmunoassay has revealed significant increases during the perimenstrual phase (Ohbuchi et al., 1995, Salamonsen et al., 1992). ET-1 has been immunolocalised to the SE, GE, vascular endothelial cells throughout the cycle, with positive staining of stromal cells during the perimenstrual period only (Ohbuchi et al., 1995). In situ hybridisation studies have confirmed these findings with ET-1 mRNA identified in epithelial and endothelial cells, as well as decidualised, perivascular stromal cells present during the late secretory phase (Salamonsen et al., 1999). Inhibition of the passage of proteins through the Golgi apparatus with the ionophore monensin resulted in an accumulation of ET-1 in GE and endothelial cells but not stromal cells, suggesting endometrial ET-1 synthesis occurs primarily in GE and endothelial cells (Salamonsen et al., 1992).

Neutral endopeptidase (NEP) is a membrane-bound endopeptidase that has the ability to degrade ET-1. NEP has been identified in the human endometrium, mainly in the stromal compartment of the functional layer, and its level varies across the menstrual cycle (Iwase et al., 2007, Head et al., 1993). Immunohistochemical staining revealed low levels in proliferative endometrium with increased staining observed during the secretory phase. Furthermore, NEP levels decrease during the late secretory phase, consistent with the possibility that ET-1 degradation is prevented at a time when vasoconstriction of the spiral arterioles is required.

The ET-1 receptors, ET_A and ET_B, have both been identified in the human endometrium (O'Reilly et al., 1992, Kubota et al., 1995). ET_A mRNA has previously been shown to be expressed throughout the menstrual cycle, whereas ET_B was increased only during menstruation (Kubota et al., 1995, O'Reilly et al., 1992). In addition, array comparison of endometrial biopsies from the mid-secretory phase to those from the late-secretory phase revealed ET_B as one of the most up-regulated genes and its significant increase was confirmed by Q-RT-PCR (Critchley et al., 2006c). This switch in ET_A/ET_B ratio at menstruation suggests ET-1 may exert different physiological effects at this time. Confirmation herein, that ET_B mRNA is

significantly increased in endometrium from the menstrual phase, lends further support to this hypothesis. Speculatively, one receptor may mediate the proliferative effects of ET-1 during the first half of the cycle and a switch in receptor signalling might promote more potent vasoconstrictive and angiogenic properties perimenstrually. However, the functional relevance of these receptor expression patterns remains to be established.

3.4.6 Summary

Data presented in this chapter confirm and extend existing literature on repair factors expresion in the human endometrium. Importantly, this observational study of well characterised human endometrium included samples from the menstrual phase, a prerequisite when investigating repair factors and their receptors. Expression of IL-8, AM, VEGF, CTGF and ET-1 mRNA was significantly increased during the menstrual phase. In addition, protein levels of IL-8, AM, VEGF and CTGF were found to have maximal secretion or immunostaining during the menstrual or proliferative phases. Receptor mRNA expression was identified in human endometrial samples for IL-8, AM, VEGF, and ET-1 and displayed significant variations across the cycle. These findings support a physiological role for these factors in the human endometrium, in particular during the perimenstrual phase when endometrial repair commences.

4. The Role of Prostaglandins and Hypoxia in the Regulation of Endometrial Repair Factors

4.1 Introduction

Throughout the menstrual cycle, the endometrial endocrine environment changes significantly (Chapter 1, Figure 1). The cyclical production of ovarian hormones, i.e. oestrogen and progesterone, controls endometrial breakdown and repair.

Oestrogen is the dominant hormone of the proliferative phase and was traditionally thought to initiate post-menstrual repair and govern subsequent endometrial remodelling. However, the mouse model of simulated menstruation suggests full restoration of the endometrium can occur in the absence of oestrogen (Kaitu'u-Lino et al., 2007a). Findings in the ovariectomised macaque model are also consistent with an oestrogen independent phase of repair (Nayak and Brenner, 2002). Furthermore, the results contained in Chapter 3 of this thesis detail significant increases in putative repair factors at the time of menstruation. Using scanning electron microscopy, Ludwig and Spornitz demonstrated that epithelial cell proliferation and migration commenced on day two of menstruation (Ludwig and Spornitz, 1991). As oestrogen levels remain low throughout the menstrual phase, these observations also suggest oestrogen is not essential for early endometrial repair. Therefore, pre-menstrual endometrial events are more likely to initiate the repair process than post-menstrual increases in oestrogen.

After ovulation, the endometrium is exposed to high levels of progesterone, secreted from the corpus luteum. In the absence of pregnancy the corpus luteum regresses and progesterone levels fall dramatically. It is well established that this progesterone withdrawal initiates a cascade of inflammatory mediators that culminates in tissue destruction and menstruation, reviewed in (Jabbour et al., 2006). This chapter tests the hypothesis that progesterone withdrawal, and its downstream effects, increase factors involved in endometrial repair. Previous studies have shown that progesterone withdrawal leads to up-regulation of endometrial COX-2 and subsequent increased synthesis of prostaglandins, namely prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Critchley et al., 1999, Sugino et al., 2004). The role of prostaglandins in the pre-menstrual endometrium is not fully understood. $PGF_{2\alpha}$ is

known to induce myometrial contractions and vasoconstriction of the endometrial spiral arterioles. This is thought to lead to an episode of local, transient hypoxia in the uppermost endometrial zones during menstruation. This hypoxic episode was first observed in classic experiments in the rhesus monkey (Markee, 1940) and was recently detected in the mouse model of menstruation (Fan et al., 2008). Use of pimonidazole, a marker of oxygen levels below 10mmHg, demonstrated intense hypoxia in the luminal portion of mouse endometrium on day 2 of the cycle, with negligible detection of pimonidazole by day 5. In addition, markers of tissue hypoxia (CAIX and HIF-1α) have been detected immunohistochemically in the human endometrium during menstruation (Critchley et al., 2006b, Punyadeera et al., 2006).

The data presented in this chapter detail the effects of progesterone withdrawal, hypoxia, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ on endometrial repair factor expression, using IL-8, VEGF, AM and CTGF as examples. This work is carried out (i) *in vitro* on primary endometrial stromal cells and a human endometrial epithelial cell line (ii) *ex vivo* in human endometrial tissue explants and (iii) using an *in vivo* model of progesterone "deprivation".

4.2 Methods

4.2.1 Tissue collection

Human endometrial biopsies were collected with ethical approval and written informed consent as described in Chapter 2.1. The samples used for *in vitro* culture herein were categorised as proliferative, early-mid or late secretory, based on LMP, histological dating and serum ovarian hormone levels (Table 6).

Table 6. Details of endometrial biopsies used for culture experiments in Chapter 4.

Stage of Cycle	Number of biopsies	Mean serum oestradiol levels, pmol/l (range)	Mean serum progesterone, nmol/l (range)
Proliferative	6	419.68 (123-739)	6.72 (2.0-13.5)
Early-Mid Secretory	10	467.86 (294-746)	51.85 (16.1-262.1)
Late Secretory	2	244.45 (168-320)	13.81 (8.7-18.9)

In addition, 12 women provided two endometrial biopsies; one collected immediately before levonorgestrel-releasing intrauterine system (LNG-IUS) insertion and the second 3-6 months after insertion (Chapter 2.1). LNG-IUS insertion dramatically down regulates endometrial progesterone receptors and provides an *in vivo* model of progesterone "deprivation" (Critchley et al., 1998). Pre-insertion biopsies were categorised into menstrual (n=1), proliferative (n=4), early- (n=5), mid- (n=1) and late-secretory (n=1) stage.

4.2.2 Cell culture

4.2.2.1 Human stromal cell extraction

Stromal cells were isolated from human endometrial tissue samples from the proliferative (n=3) and secretory (n=3) phases as described in Chapter 2.3.2.

4.2.2.2 Transfection of endometrial epithelial cells

Human Ishikawa endometrial adenocarcinoma cells previously stably transfected with a PGE_2 receptor (EP2) or a $PGF_{2\alpha}$ receptor (FP) were used for *in vitro* experiments. Details of transfection and maintenance can be found in Chapter 2.3.3.

4.2.2.3 Treatment of cells *in vitro*

Endometrial cells were seeded in 6 well plates in media supplemented with 10% fetal calf serum (Table 7). The following day, cells were washed in PBS and incubated in serum-free culture medium containing antibiotics and 8.4μM indomethacin for at least 16h. Cells were treated with vehicle, 100nM PGE_2 (EP2S cells/HES) or 100nM $PGF_{2\alpha}$ (FPS cells/HES) and placed in either normoxia (37°C, 21% O_2, 5% CO_2) or hypoxic conditions (37°C, 0.5% O_2, 5% CO_2) in a sealed chamber (Coy laboratory products, Michigan, USA) for 2, 4, 8, 24 and 48 h. Culture supernatant was collected and stored at -20°C. Cells were washed in PBS and RNA extracted and stored at minus 80°C.

Table 7. Details of culture conditions for *in vitro* cell studies in Chapter 4.

Cell Type	No. of cells per well	Media
Human Endometrial Stromal (HES)	3×10^5	RPMI 1640
Ishikawa EP2S	4×10^5	DMEM F-12 with glutamax-1 and pyridoxine
Ishikawa FPS	4×10^5	DMEM F-12 with glutamax-1 and pyridoxine

4.2.2.4 Hypoxia and prostaglandin treatments

All endometrial biopsies (Table 6) were divided into four equal explants and incubated on raised platforms in 24 well plates, just covered with serum free RPMI 1640 medium and 8.4μM indomethacin for 16h.

Explants were then treated for 24h as detailed in Table 8, where normoxia was incubation at 21% O_2, 5% CO_2, 37°C and hypoxia was incubation in a sealed hypoxic chamber (Coy laboratory products) set at 0.5% O_2, 5% CO_2, 37°C.

Table 8. Treatment of endometrial tissue biopsies with prostaglandins (PG) in normoxic and hypoxic conditions.

Number of biopsies		**Treatments**			
Proliferative	Secretory	Explant 1	Explant 2	Explant 3	Explant 4
3	5	Normoxia (21% O_2)		Hypoxia (0.5% O_2)	
		ETOH vehicle	100nM PGE_2	ETOH vehicle	100nM PGE_2
3	3	Normoxia (21% O_2)		Hypoxia (0.5% O_2)	
		ETOH vehicle	100nM $PGF_{2\alpha}$	ETOH vehicle	100nM $PGF_{2\alpha}$

4.2.2.5 Ex vivo model of progesterone antagonism

Five endometrial biopsies from the proliferative phase were divided into eight equal sized explants and placed on raised platforms in four wells of two 12 well plates. All explants were treated with 1μM medroxyprogesterone acetate (MPA) for 24h. Explants were then treated with:

- Explant 1: 1μM MPA plus vehicle
- Explant 2: 1μM MPA plus 8.4μM indomethacin (a COX enzyme inhibitor)
- Explant 3: 1μM MPA and 1μM RU486 (a progesterone receptor antagonist, to simulate progesterone withdrawal) plus vehicle
- Explant 4: 1μM MPA and RU486 plus 8.4μM indomethacin, to prevent prostaglandin production.

Immediately after these treatments, one plate was placed in normoxic conditions and the other in hypoxic conditions for 48h, as menstruation occurs 48h after progesterone withdrawal *in vivo*.

4.2.2.6 Ex vivo model of progesterone withdrawal

Four further endometrial biopsies were divided into four equal explants, placed on raised platforms in two wells of two 12 well plates. All were treated with 1μM progesterone for 24h. Explants were then washed twice with PBS and treated with either 1μM progesterone or with vehicle (control media) to induce progesterone withdrawal. After these treatments plates were placed in normoxic (21% O_2) or hypoxic (0.5% O_2) conditions for 48h.

4.2.3 RNA extraction and Q-RT-PCR

RNA was extracted from all the endometrial samples, cDNA prepared and Q-RT-PCR performed as detailed in Chapter 2.4. The expression of mRNA was measured for IL-8, VEGF, AM and CTGF (sequences detailed in Chapter 2, Table 3). A sample of liver cDNA was also prepared and included on each Taqman plate as a positive control. There was no significant change in 18S RNA expression between samples incubated in normoxic and hypoxic conditions.

4.2.4 ELISA

Protein content (IL-8, VEGF, AM, CTGF) in the culture supernatant from cell culture experiments was measured by ELISA, as detailed in Chapter 2.7. Time points were selected subsequent to the time of maximal mRNA expression, to allow for protein translation and secretion into culture supernatants.

4.2.5 Immunohistochemistry

IL-8 and VEGF proteins were localised in endometrial sections from women pre- and post- LNG-IUS insertion using the protocols described in Chapter 2.6.

4.2.6 Statistical analysis

For mRNA in cell culture and tissue explants, results were expressed as fold increase, for which relative expression of mRNA in treated samples was divided by the relative expression in vehicle-treated samples. Data are presented as mean +/- SEM. Significant difference was determined using one-way ANOVA of dCt values, with Tukey's post test analysis. Statistically significant differences in protein levels were also determined using one-way ANOVA with Tukey's post test analysis. For endometrial biopsies from women pre- and post- LNG-IUS insertion, mRNA results were expressed as quantity relative to a comparator, a sample of RNA from the liver. Significant difference was determined using a Wilcoxon matched pairs test (GraphPad Prism Software, Inc., San Diego, CA).

4.3 Results

4.3.1 Interleukin-8 (IL-8)

4.3.1.1 PGE_2 and hypoxia increased IL-8 mRNA in EP2S cells

To investigate the role of PGE_2 and hypoxia in the regulation of IL-8 expression, an Ishikawa endometrial epithelial cell line stably expressing the EP2 receptor was utilised (EP2S cells). This cell line was used to mimic primary endometrial epithelial cells, which express receptors for PGE_2 (Milne et al., 2001) but do not proliferate well in culture. Cells were exposed to vehicle treatment or 100nM PGE_2 for up to 48 hours in normoxic and hypoxic conditions. Treatment with PGE_2 in normoxic conditions (Figure 31A) showed a significant increase in IL-8 mRNA, with maximal up-regulation after 8 hours ($p<0.01$). Hypoxic conditions also significantly increased IL-8 mRNA (Figure 31B) but displayed a more delayed induction, reaching maximum up-regulation after 8-24 hours ($p<0.01$).

4.3.1.2 Simultaneous treatment with PGE_2 and hypoxia revealed a synergistic increase in IL-8 mRNA and protein in EP2S cells

When EP2S cells were exposed to both PGE_2 and hypoxic conditions for 24 hours (Figure 32A), there was a synergistic increase in IL-8 mRNA that was significantly greater than treatment with PGE_2 in normoxia ($p<0.05$) or hypoxic conditions alone ($p<0.05$). Levels of secreted IL-8 protein showed a similar pattern, with a synergistic increase in IL-8 protein secretion with PGE_2 treatment in hypoxic conditions (Figure 32B).

4.3.1.3 $PGF_{2\alpha}$ and hypoxia increased IL-8 mRNA in FPS cells

To examine the contribution of $PGF_{2\alpha}$ to the regulation of IL-8, Ishikawa cells stably transfected with the $PGF_{2\alpha}$ receptor were examined (FP2S cells) to mimic primary endometrial epithelial cells, which express the FP receptor *in vivo* (Milne and Jabbour, 2003). Treatment of FPS cells with 100nM $PGF_{2\alpha}$ for 8h revealed a significant increase in IL-8 mRNA versus vehicle treated cells ($p<0.01$). This increase was not significantly enhanced by the addition of hypoxic conditions (Figure 33A).

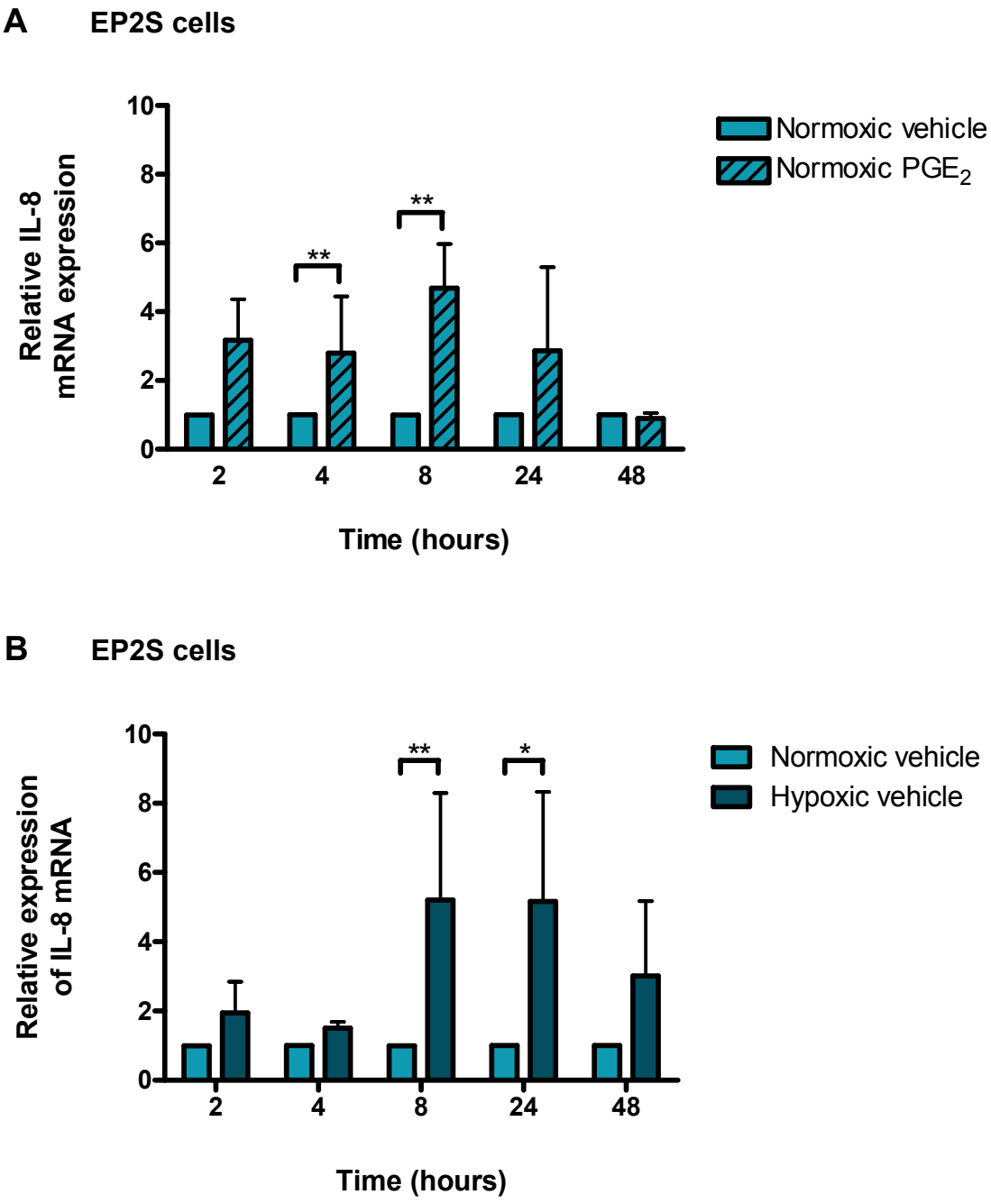


Figure 31. **The regulation of IL-8 mRNA in EP2S cells. (A)** Cells treated with vehicle or 100nM prostaglandin E_2 (PGE_2) for up to 48h (n=3). **(B)** Cells treated in normoxia (21% O_2) or hypoxia (0.5% O_2) for up to 48h (n=3). (*$p<0.05$, **$p<0.01$).

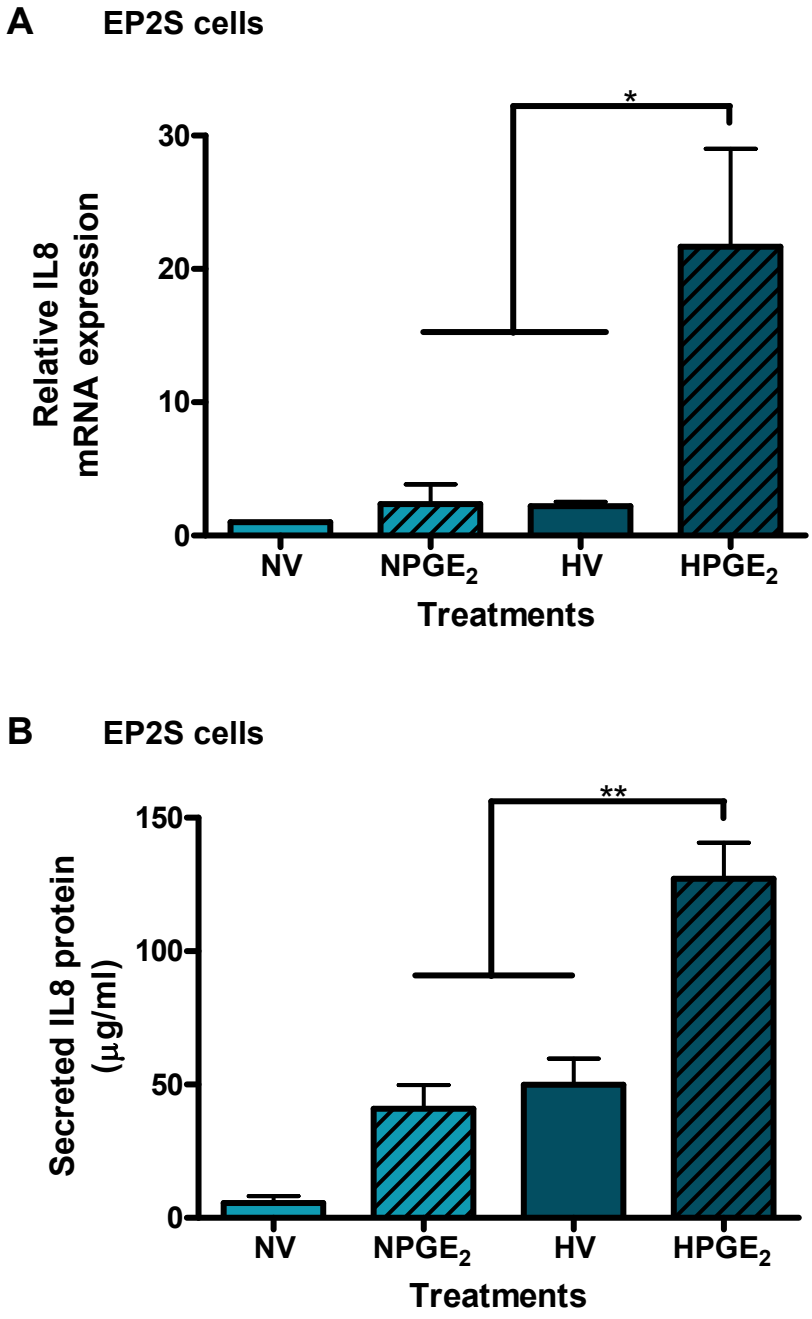


Figure 32. The effect of simultaneous treatment of endometrial epithelial cells (EP2S cells) with 100nM prostaglandin E_2 (PGE_2) and hypoxia for 24h on **(A)** IL-8 mRNA expression (n=3) and **(B)** IL-8 secreted protein levels (n=3). N: normoxia 21% O_2, H: hypoxia 0.5% O_2, V: ethanol vehicle. (*$p<0.05$, **$p<0.01$).

Treatment of FPS cells with $PGF_{2\alpha}$ and/or hypoxic conditions for 24h had no significant impact on IL-8 expression (Figure 33B). IL-8 secreted protein was significantly increased by $PGF_{2\alpha}$ at 24h when compared to control cells ($p<0.05$) but was not significantly increased by hypoxia (Figure 33C).

4.3.1.4 PGE_2 increased IL-8 mRNA in human endometrial stromal (HES) cells

Treatment of HES cells with 100nM PGE_2 resulted in a significant increase in IL-8 mRNA after 48h ($p<0.05$) (Figure 34A). HES cells exposed to hypoxic conditions showed no significant increase in IL-8 mRNA at any time-point examined (Figure 34B).

4.3.1.5 Simultaneous treatment of HES cells with PGE_2 and hypoxia showed no synergistic increase in IL-8 mRNA or protein

Based on the timecourse above, HES cells were treated with 100nM PGE_2 in hypoxic conditions for 48h and showed a significant increase in IL-8 mRNA ($p<0.05$). However, this was not significantly different to treatment with PGE_2 in normoxic conditions (Figure 35A). IL-8 secreted protein levels from HES cells after 48h of culture were not significantly changed by any treatment (Figure 35B).

4.3.1.6 $PGF_{2\alpha}$ and hypoxia have no significant impact on IL-8 mRNA and protein levels in HES cells

100nM $PGE_{2\alpha}$ and hypoxic conditions had no significant effect on HES cell IL-8 mRNA (Figure 36A). HES cells treated with 100nM $PGF_{2\alpha}$ in normoxic or hypoxic conditions had no significant increases in IL-8 protein versus vehicle treated cells in normoxia (Figure 36B).

4.3.1.7 IL-8 mRNA was increased by PGE_2 and hypoxia in secretory endometrial tissue

To investigate the regulation of endometrial IL-8, human endometrial explants were cultured for 24 hours with vehicle, 100nM PGE_2 or hypoxic conditions. Secretory endometrium from seven women showed a non-significant increase in IL-8 expression with PGE_2 treatment in normoxia. Culture of endometrial explants in

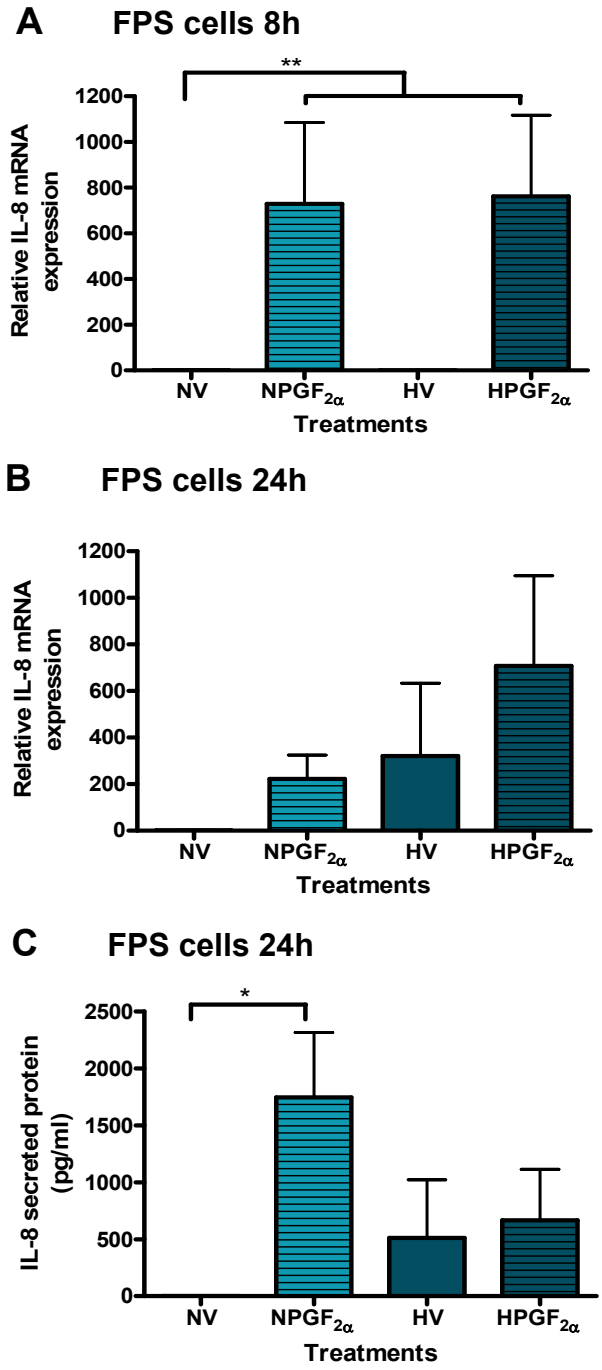


Figure 33. **The regulation of IL-8 by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and hypoxia in FPS cells. (A)** IL-8 mRNA in FPS cells treated with vehicle, 100nM $PGF_{2\alpha}$, hypoxic conditions (0.5% O_2) or both $PGF_{2\alpha}$ and hypoxia for 8h (n=3). **(B)** IL-8 mRNA in FPS cells treated in an identical manner for 24h (n=3). **(C)** IL-8 secreted protein levels from FPS cells treated for 24h (n=3). N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle. (*$p<0.05$, **$p<0.01$).

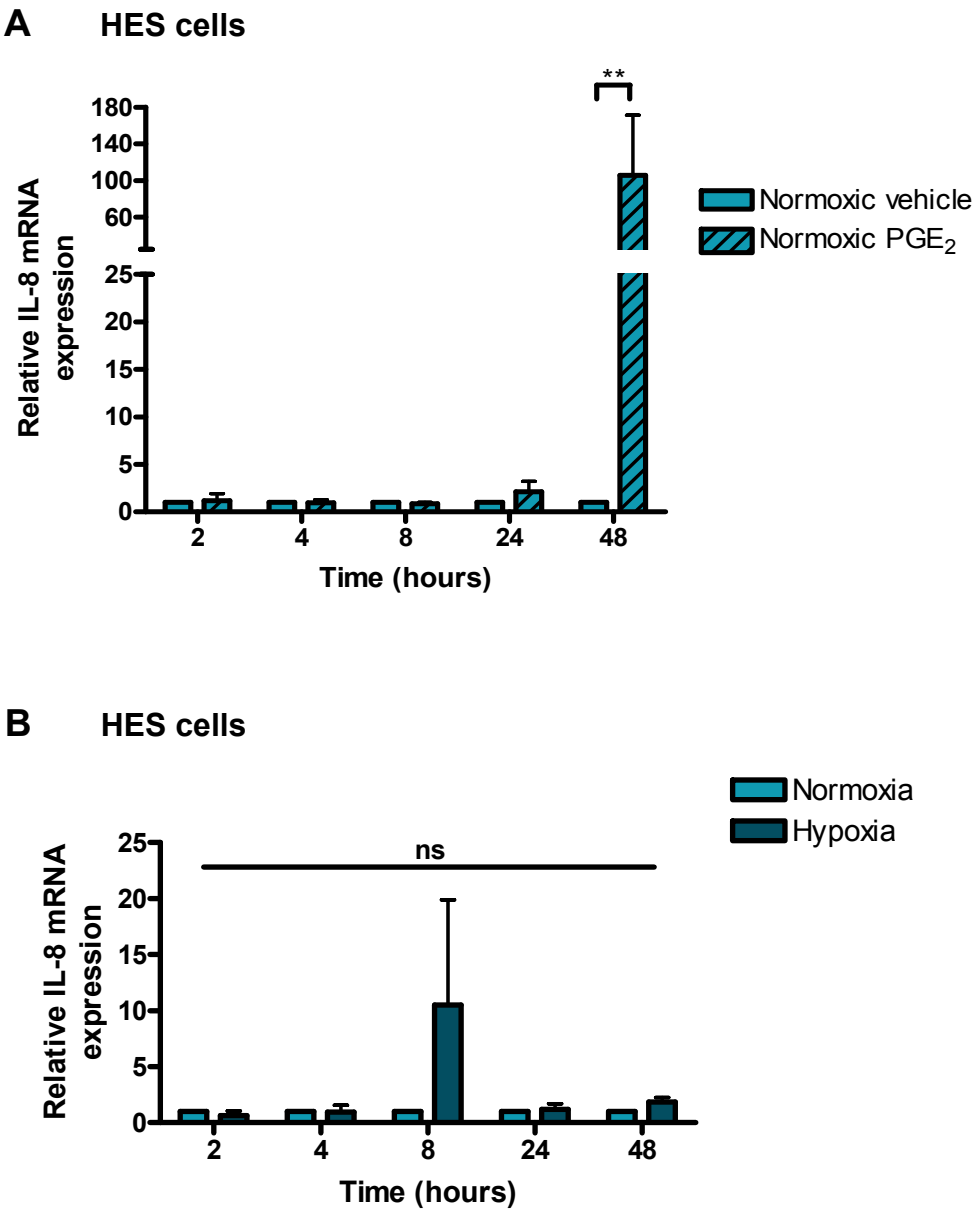


Figure 34. **The effect of prostaglandin E_2 (PGE_2) and hypoxia on IL-8 mRNA in human endometrial stromal cells (HES). (A)** IL-8 expression in HES cells treated with 100nM PGE_2 for up to 48h (n=3). **(B)** IL-8 expression in HES cells treated in normoxia or hypoxia for up to 48h (n=3). V: vehicle, N: normoxia (21% O_2), H: hypoxia (0.5% O_2). (**$p<0.01$, ns: non-significant).

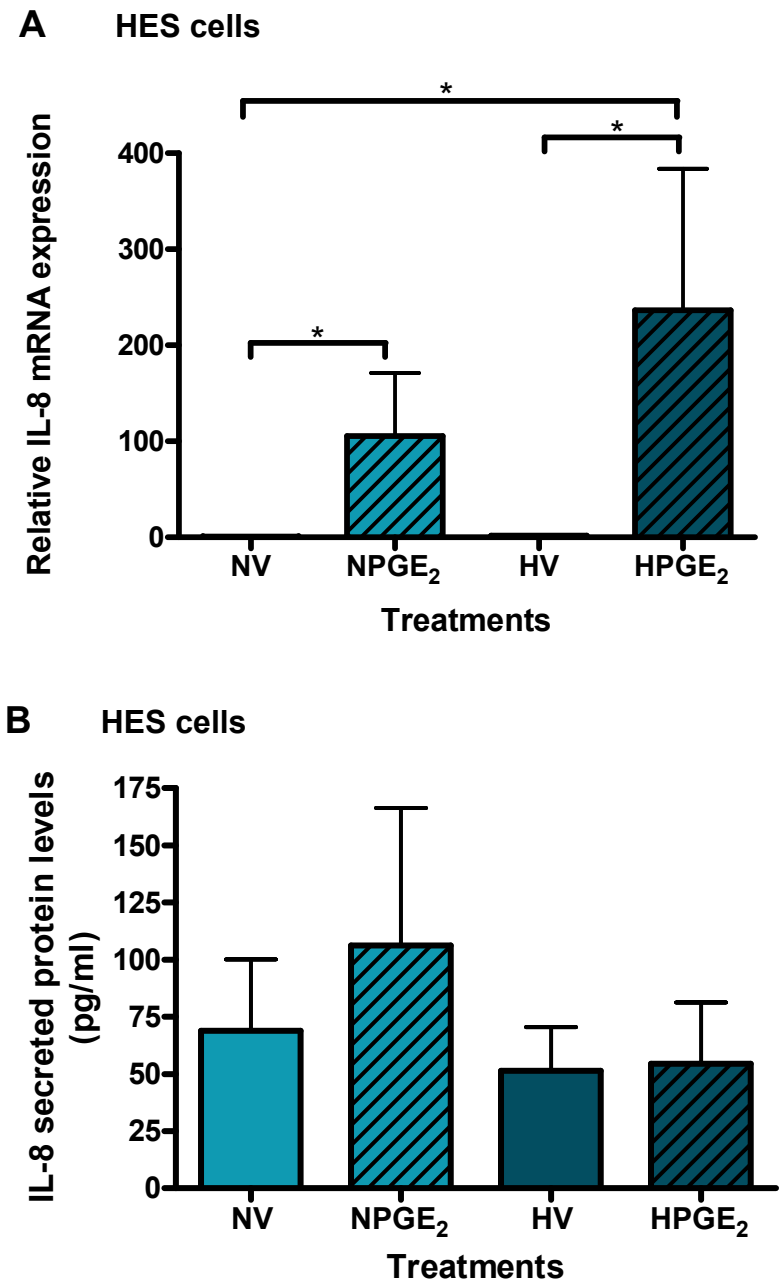


Figure 35. The effect of simultaneous treatment of human endometrial stromal cells with prostaglandin E_2 (PGE_2) and hypoxic conditions for 48h on **(A)** IL-8 mRNA expression (n=3) and **(B)** IL-8 secreted protein levels (n=3). V: vehicle treatment, N: normoxia (0.5% O_2), H: hypoxia (0.5% O_2). (*$p<0.05$).

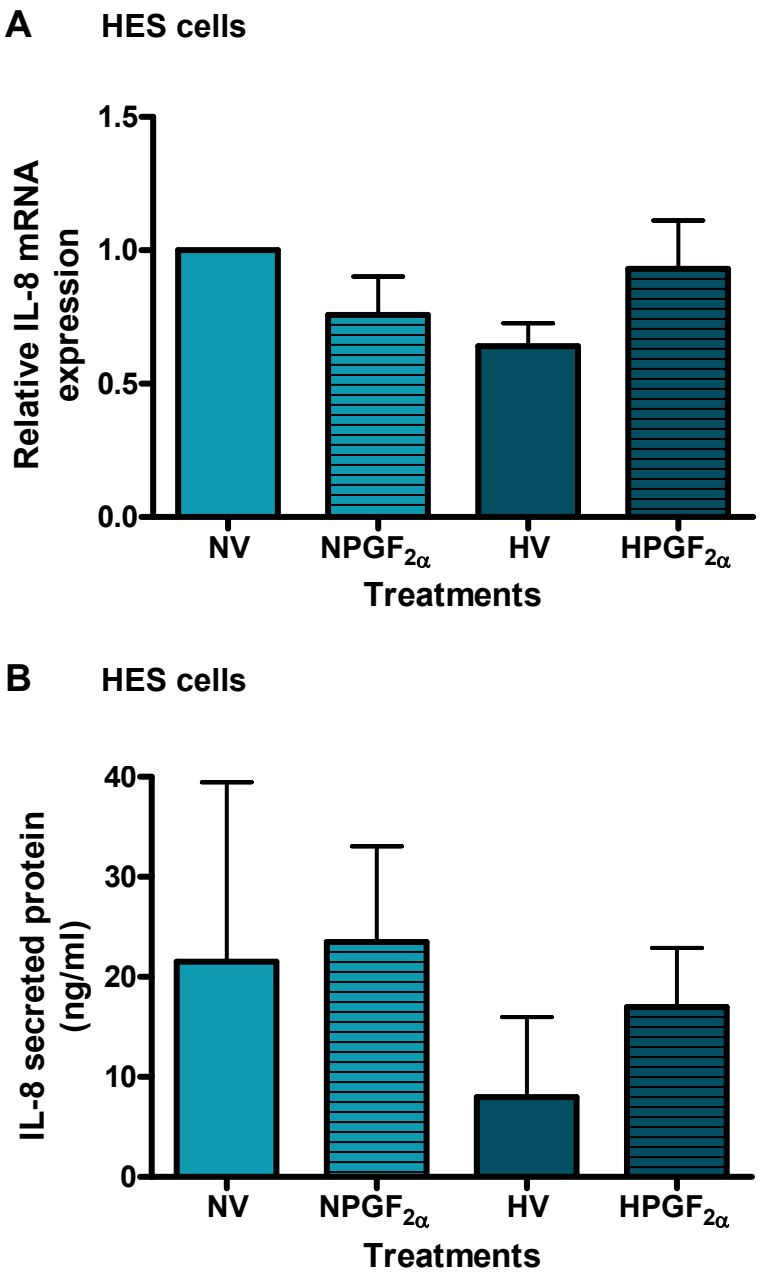


Figure 36. **The regulation of IL-8 by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and hypoxia in human endometrial stromal (HES) cells. (A)** IL-8 mRNA in HES cells treated with vehicle, 100nM $PGF_{2\alpha}$, hypoxic conditions (0.5% O_2) or both $PGF_{2\alpha}$ and hypoxia for 24h (n=3). **(B)** IL-8 secreted protein levels from HES cells treated in an identical manner for 24h (n=3). N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle. (ns: non-significant).

hypoxic conditions significantly elevated IL-8 mRNA ($p<0.05$) (Figure 37B). In contrast, neither treatment induced up-regulation of IL-8 in endometrium from the proliferative phase (n=3) (Figure 37A). This suggests previous exposure to progesterone is essential for up-regulation of IL-8 by PGE_2 and hypoxia.

4.3.1.8 IL-8 mRNA was not significantly increased by $PGF_{2\alpha}$ in endometrial tissue

Treatment of proliferative or secretory endometrial tissue explants with 100nM $PGF_{2\alpha,}$ hypoxic conditions or both $PGF_{2\alpha}$ and hypoxia did not significantly increase IL-8 mRNA versus explants treated with vehicle in normoxia (Figure 38A,B).

4.3.1.9 An *in vitro* model of progesterone-antagonism increased IL-8 mRNA expression.

To establish if progesterone withdrawal induces endometrial IL-8 mRNA, proliferative endometrial biopsies were divided into 8 explants (n=5). All explants were treated with medroxyprogesterone acetate for 24hours. Following progesterone exposure, progesterone withdrawal was simulated in four of the explants by co-treating with RU486, a progesterone receptor antagonist. Progesterone withdrawal in normoxic conditions did not significantly up-regulate IL-8 mRNA (Figure 39). *In vivo,* it is postulated that progesterone withdrawal in the late secretory phase induces synthesis of prostaglandins and constriction of spiral arterioles, resulting in an episode of transient hypoxia. Therefore, to mimic the *in vivo* situation more accurately, two of the endometrial explants were exposed to hypoxic conditions at the time of simulated progesterone withdrawal. Addition of hypoxic conditions induced a significant induction of IL-8 mRNA 48h after progesterone withdrawal ($p<0.05$) (Figure 39).

To assess the contribution of prostaglandins following progesterone withdrawal, we co-treated explants with MPA (progestogen), RU486 (progesterone receptor antagonist) and indomethacin (a COX enzyme inhibitor). Addition of indomethacin attenuated the up-regulation of IL-8 mRNA following progesterone withdrawal in hypoxic conditions (Figure 39).

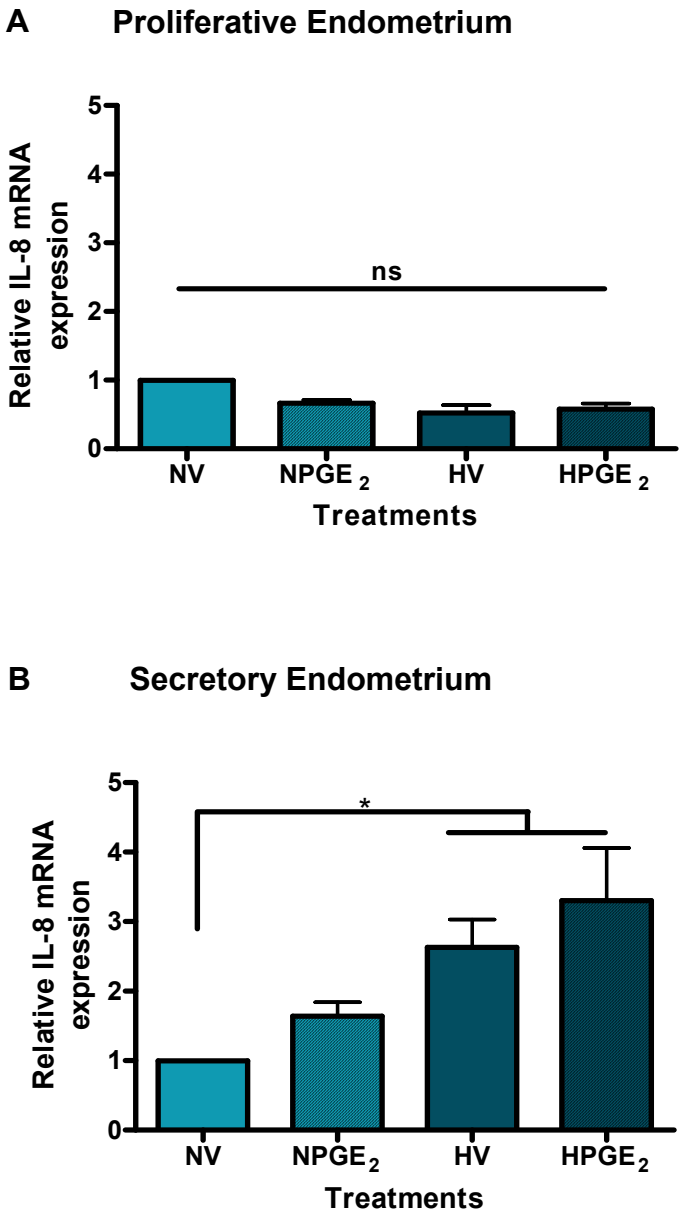


Figure 37. **The effect of 100nM prostaglandin E_2 and hypoxia on IL-8 mRNA expression in endometrial tissue explants**. **(A)** Endometrial explants from the proliferative phase (n=3) treated with vehicle, 100nM PGE_2, hypoxia or both PGE_2 and hypoxia for 24h. **(B)** Endometrial explants from the secretory phase (n=5) treated in an identical manner. N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle. (*$p<0.05$).

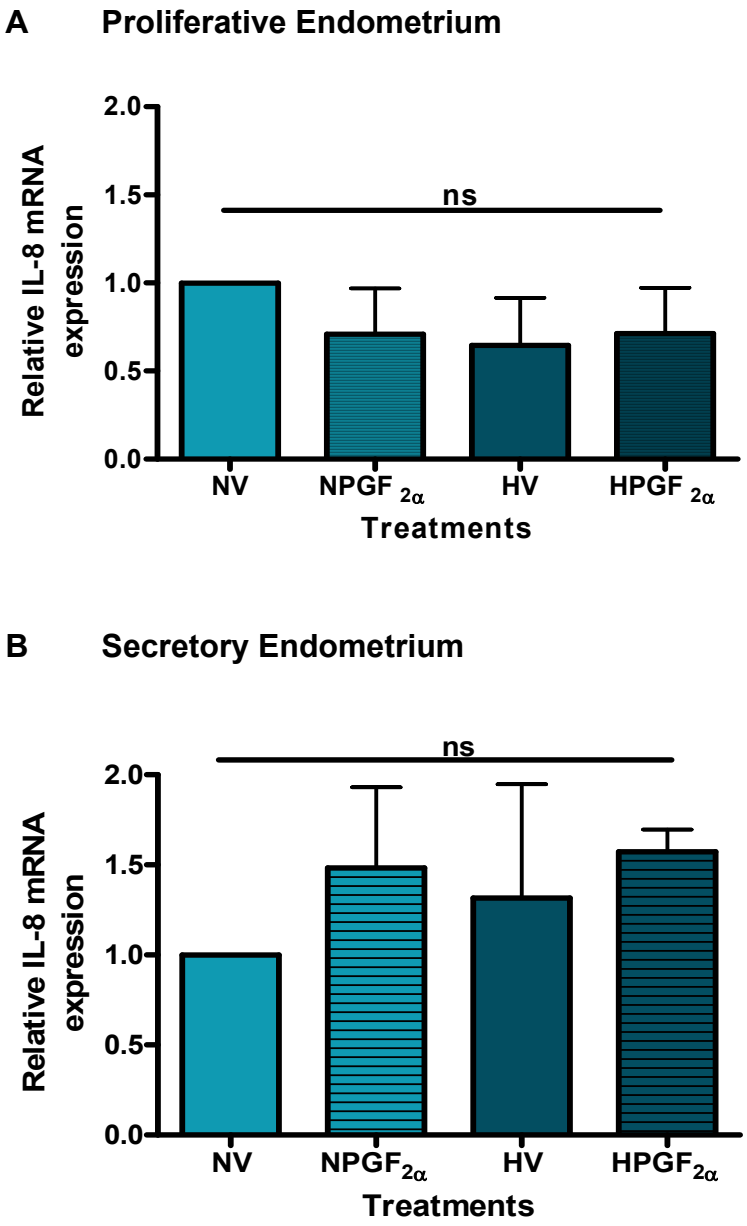


Figure 38. **The effect of 100nM prostaglandin $F_{2\alpha}$ and hypoxia on IL-8 mRNA in endometrial tissue explants**. **(A)** Endometrial explants from the proliferative phase (n=3) treated with vehicle, $PGF_{2\alpha}$, hypoxia or both $PGF_{2\alpha}$ and hypoxia for 24h. **(B)** Endometrial explants from the secretory phase (n=3) treated in an identical manner. N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle. (ns: non-significant).

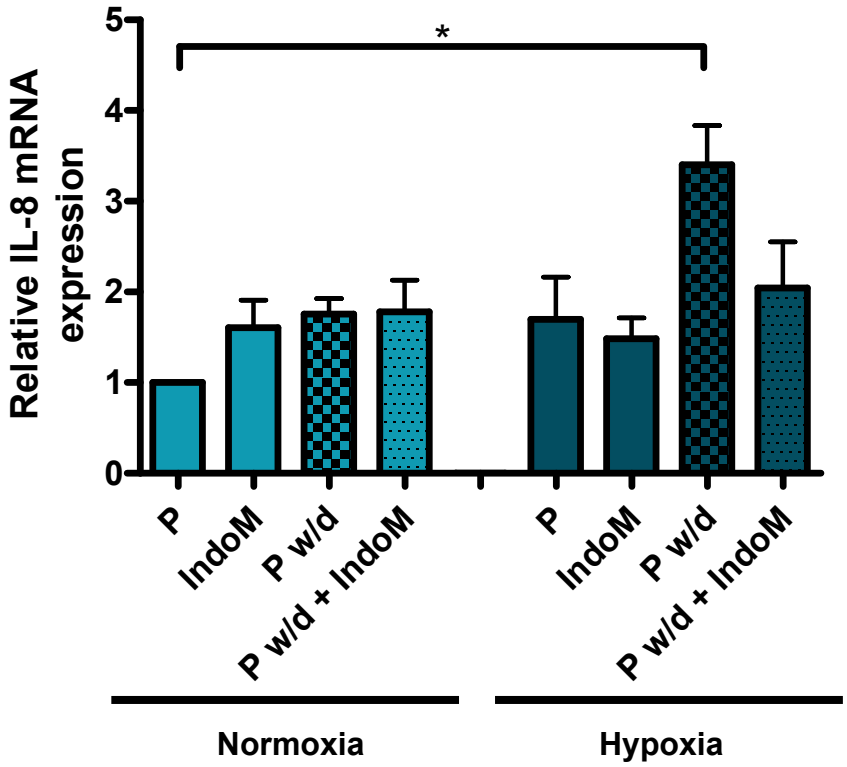


Figure 39. **The effect of *in vitro* progesterone antagonism on endometrial IL-8 mRNA expression**. Proliferative phase explants (n=4) were pre-treated with 1μM medroxyprogesterone acetate (MPA) and then (i) maintained in 1μM MPA (P), (ii) maintained in MPA plus 8.4μM indomethacin, a COX enzyme inhibitor (P+IndoM), (iii) co-treated with 1μM MPA and 1μM RU486, a progesterone receptor antagonist (Pw/d) or (iii) co-treated with 1μM MPA, 1μM RU486 and 8.4μM indomethacin (Pw/d + IndoM). Identical treatments were incubated in either normoxic (21% O2) or hypoxic (0.5% O2) conditions for 48h.

4.3.1.10 Endometrium collected after exposure to LNG-IUS had increased expression of IL-8 mRNA compared to pre-insertion biopsies

To further investigate the role of progesterone and hypoxia in regulating endometrial IL-8 expression, endometrial biopsies were examined from nine women taken pre- and 3-6 months post- levonorgestrel-releasing intrauterine system (LNG-IUS) insertion. The LNG-IUS has been shown to markedly down-regulate the progesterone receptor in all components of the endometrium (Critchley et al., 1998), resulting in a human model of "progesterone-deficiency". There was a significant up-regulation of IL-8 mRNA after LNG-IUS exposure when compared to pre-insertion biopsies from the proliferative, early and mid secretory phases (n=7) ($p<0.05$) (Figure 40A). Interestingly, endometrial biopsies taken during the late-secretory and menstrual phases (n=2) showed no significant change in IL-8 mRNA on exposure to the LNG-IUS (Figure 40B). This suggests endometrium already exposed to progesterone withdrawal *in vivo* has no further capacity for IL-8 induction on insertion of LNG-IUS.

4.3.1.11 IL-8 immunohistochemical staining is increased in endometrium that has been exposed to LNG-IUS

This increase in endometrial IL-8 after LNG-IUS insertion was also identified at the protein level. Increased IL-8 immunohistochemical staining was visible in the decidualised stromal cells present after LNG-IUS exposure (Figure 41).

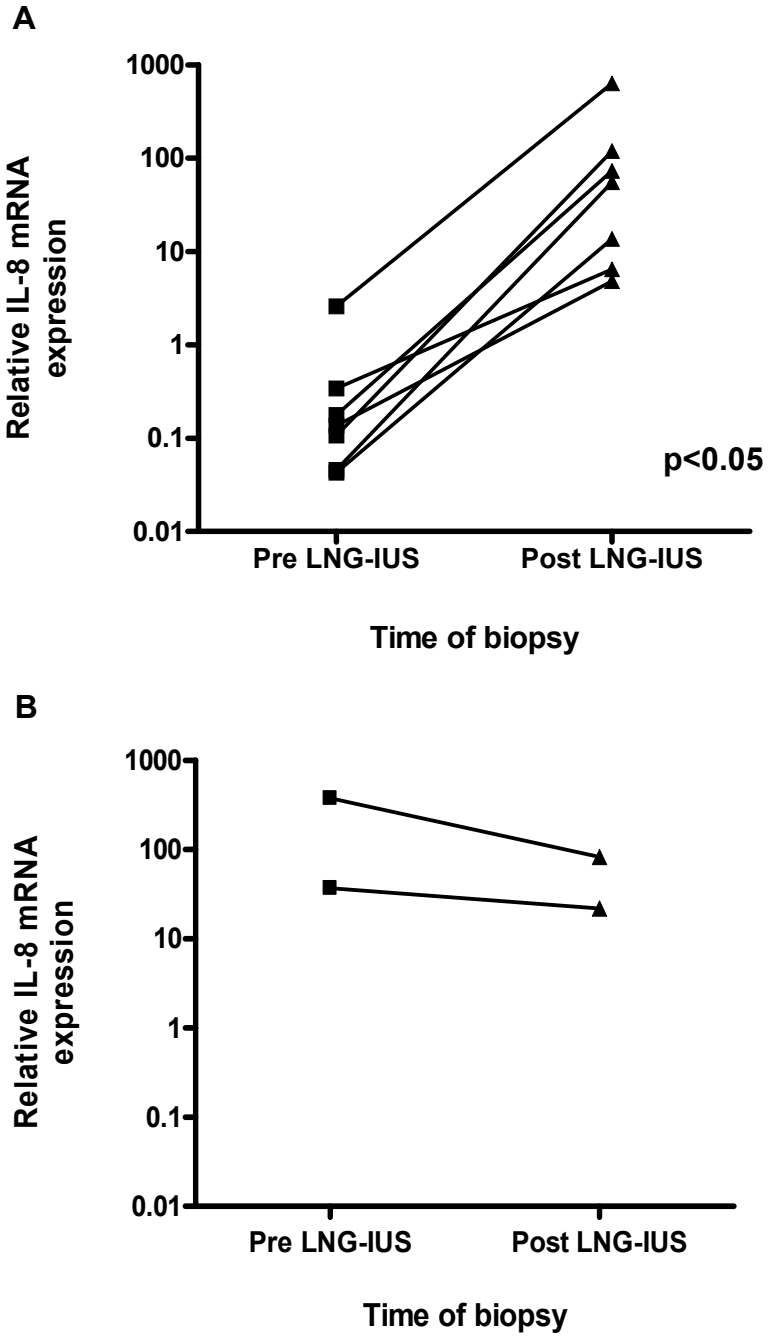


Figure 40. **The effect of a levonorgestrel releasing intra uterine system (LNG-IUS) on IL-8 mRNA**. **(A)** IL-8 mRNA in proliferative, early- and mid-secretory endometrium prior to LNG-IUS insertion was compared to paired samples collected 3-6 months after insertion. **(B)** Pre- and post-insertion samples in endometrium from the menstrual and late secretory phase. Note logarithmic scale on y-axis.

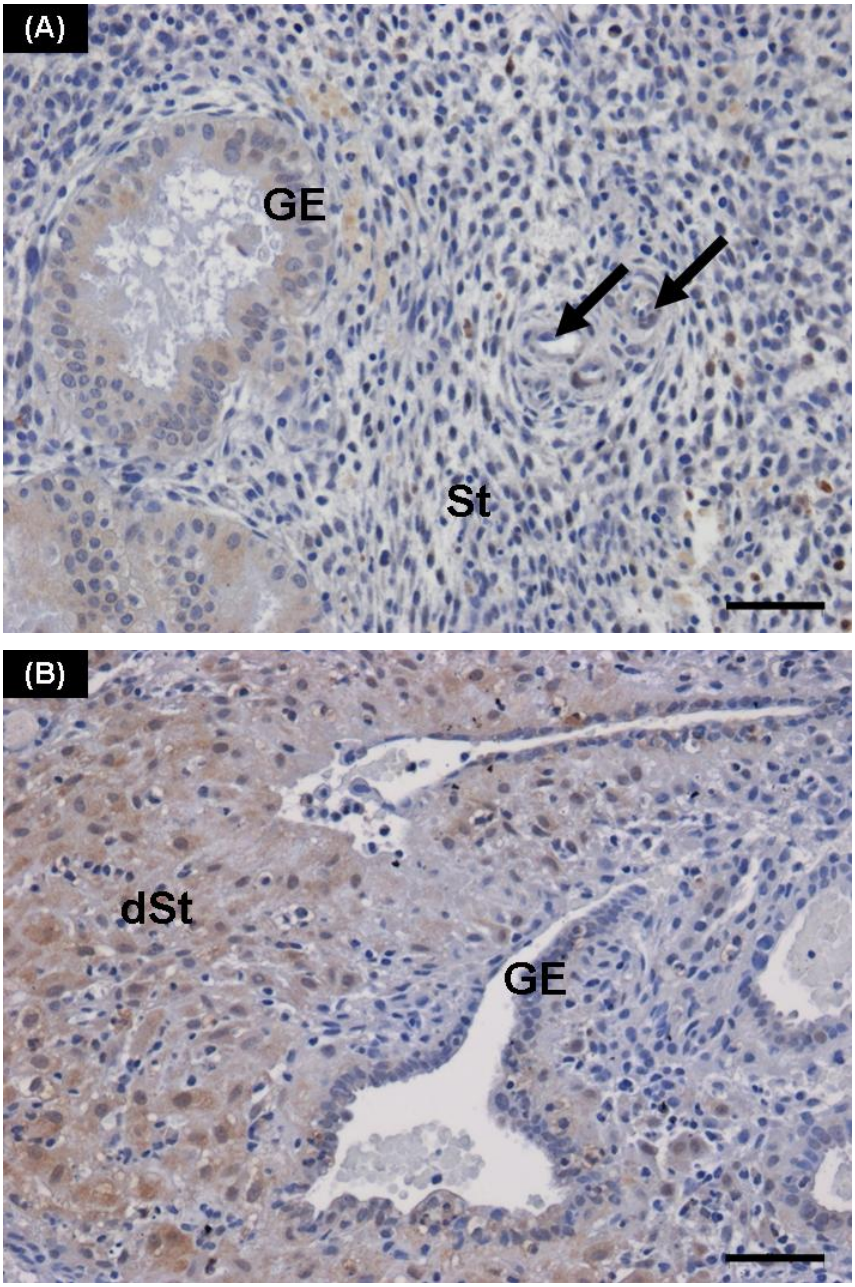


Figure 41. **Immunohistochemical staining for IL-8** in **(A)** mid-secretory endometrium prior to LNG-IUS insertion and **(B)** in endometrium from the same woman 4 months after LNG-IUS insertion. GE: glandular epithelial cells, St: stromal cell compartment, arrows = endothelial cells, dSt: decidualised stromal cells. Scale bar = 50μm.

4.3.2 Adrenomedullin (AM)

4.3.2.1 $PGF_{2\alpha}$ and hypoxic conditions up-regulate AM expression in endometrial epithelial cells

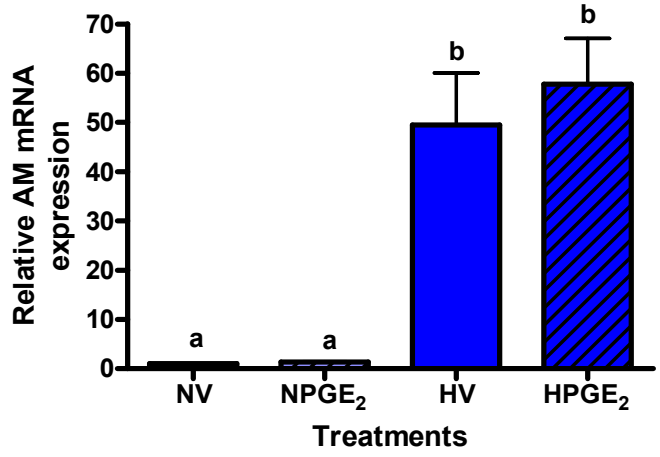
Treatment of EP2S cells with 100nM PGE_2 had no significant impact on AM expression at 8h (Figure 46A) or 2, 4, 24 or 48h (data not shown). EP2S cells placed in hypoxic conditions (0.5% O_2) displayed a significant up-regulation of AM mRNA, with a 50 fold increase over cells in normoxic conditions ($p<0.001$) (Figure 42A). There was no further increase when cells were co-treated with hypoxia and 100nM PGE_2. Treatment with 100nM $PGF_{2\alpha}$ significantly increased AM mRNA in FPS cells, resulting in a >11 fold increase over vehicle treated cells after 8h ($p<0.001$) (Figure 42B). Hypoxic conditions also significantly increased AM expression in FPS cells >6 fold ($p<0.001$). Interestingly, co-treatment of FPS cells with $PGF_{2\alpha}$ and hypoxia resulted in a synergistic increase in AM mRNA (>28 fold increase over normoxic vehicle) that was significantly greater than treatment with $PGF_{2\alpha}$ or hypoxia alone ($p<0.05$, $p<0.001$ respectively). Analysis of FPS cell culture supernatants by ELISA revealed no significant differences in AM secreted protein levels after 24 h but a similar pattern emerged, with maximal secreted AM protein from cells treated with both $PGF_{2\alpha}$ and hypoxia (Figure 42C).

4.3.2.2 $PGF_{2\alpha}$ and hypoxia up-regulate AM expression in secretory endometrial tissue

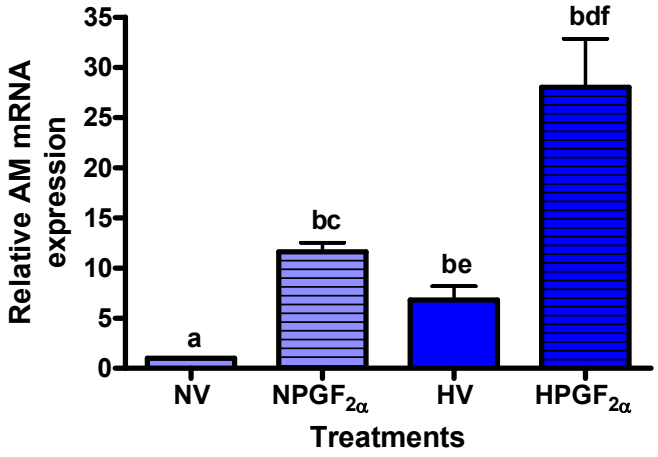
To further investigate $PGF_{2\alpha}$ and hypoxia mediated AM expression, endometrial tissue explants were treated with (i) vehicle, (ii) 100nM $PGF_{2\alpha}$, (iii) hypoxic conditions, or (iv) $PGF_{2\alpha}$ and hypoxia. Proliferative biopsies revealed no significant differences in AM mRNA with these treatments (n=3) (Figure 43A). In contrast, endometrial tissue from the secretory phase did show a significant increase in AM expression when placed in hypoxic conditions (n=4) (>4 fold, $p<0.05$) (Figure 43B). In addition, there was a non-significant two-fold increase in AM mRNA after treatment with $PGF_{2\alpha}$ in normoxia. This suggests that previous

Figure 42. **The regulation of adrenomedullin (AM) mRNA in endometrial epithelial cells. (A)** Ishikawa EP2S cells were treated with vehicle, 100nM prostaglandin E_2 (PGE_2), hypoxic conditions or both PGE_2 and hypoxia for 8h. **(B)** Ishikawa FPS cells were treated with 100nM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), hypoxia or both $PGF_{2\alpha}$ and hypoxia for 8h. **(C)** ELISA analysis of AM secreted protein levels in FPS cell culture supernatants after 24h. All experiments were carried out n=3. N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle. (a-b $p<0.001$, c-d $p<0.05$, e-f $p<0.01$).

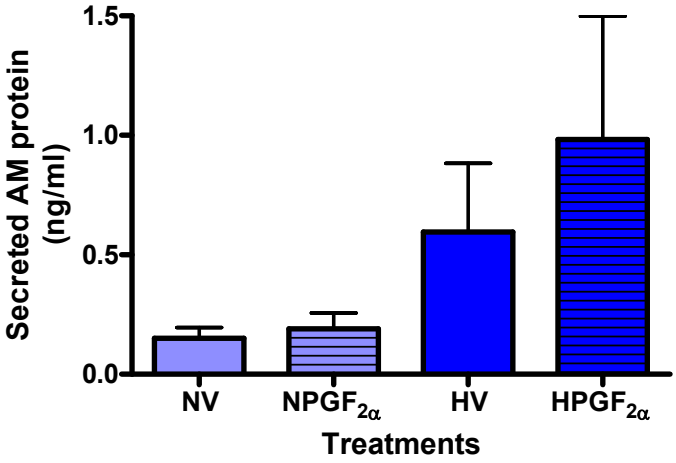
A EP2S cells



B FPS cells



C FPS cells



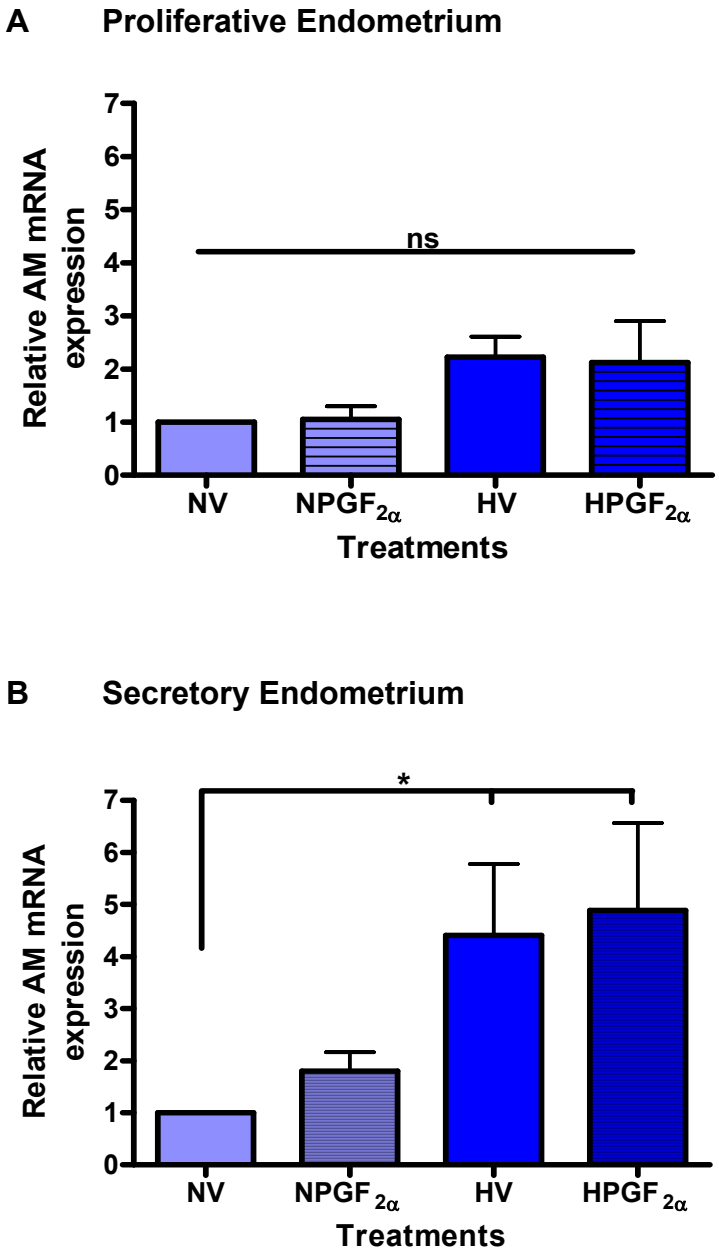


Figure 43. **Adrenomedullin (AM) mRNA in endometrial tissue explants**. **(A)** Endometrial explants from the proliferative phase treated with vehicle, 100nM $PGF_{2\alpha}$, hypoxia or both $PGF_{2\alpha}$ and hypoxia for 24h (n=3). **(B)** Secretory phase endometrial explants treated in an identical manner for 24h (n=3). N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle. (ns: non-significant, *$p<0.05$).

exposure to progesterone is a pre-requisite to enable up-regulation of AM mRNA by $PGF_{2\alpha}$ and hypoxia.

4.3.2.3 Progesterone withdrawal, in the presence of hypoxia and prostaglandins, up-regulates AM expression in proliferative endometrial tissue

To examine the contribution of progesterone exposure and withdrawal, an *in vitro* model of progesterone antagonism was used. Proliferative explants were treated with progesterone followed by progesterone and mifepristone (RU486, a progesterone receptor antagonist), to mimic progesterone withdrawal. There was no significant difference in endometrial AM mRNA in explants subjected to progesterone withdrawal when compared to those maintained in progesterone. However, when progesterone withdrawal was carried out in hypoxic conditions to simulate the *in vivo* scenario, a significant increase in AM mRNA was observed ($p<0.05$) (Figure 44). The contribution of prostaglandins, which are up-regulated downstream of progesterone withdrawal, was assessed by co-culture of explants with indomethacin, a COX enzyme inhibitor. Addition of indomethacin resulted in abrogation of the AM increase observed following progesterone withdrawal in hypoxic conditions (Figure 44). These data suggest that progesterone withdrawal, with subsequent prostaglandin production and induction of hypoxia, results in up-regulation of AM mRNA at menstruation.

4.3.2.4 Endometrium collected after LNG-IUS exposure had increased AM mRNA versus pre-insertion biopsies

Endometrial biopsies were performed prior to and 3-6 months after LNG-IUS insertion. Biopsies taken after LNG-IUS insertion had significantly increased AM mRNA when compared to pre-insertion biopsies taken during the proliferative and early-mid secretory phases ($p<0.05$) (Figure 45).

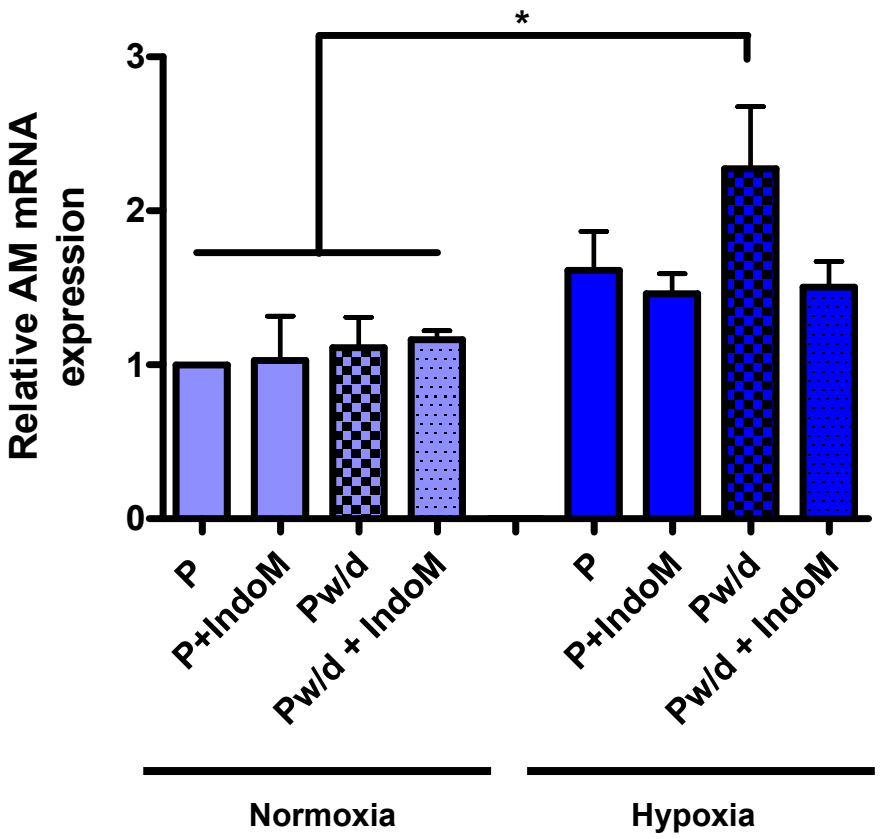


Figure 44. **The effect of *in vitro* progesterone antagonism on endometrial adrenomedullin (AM) mRNA**. Proliferative phase explants (n=4) were pre-treated with1μM medroxyprogesterone acetate (MPA) and then (i) maintained in 1μM MPA (P), (ii) maintained in MPA plus 8.4μM indomethacin, a COX enzyme inhibitor (P+IndoM), (iii) co-treated with 1μM MPA and 1μM RU486, a progesterone receptor antagonist (Pw/d) or (iii) co-treated with 1μM MPA, 1μM RU486 and 8.4μM indomethacin (Pw/d + IndoM). Identical treatments were incubated in either normoxic (21% O2) or hypoxic (0.5% O2) conditions for 48h. (*$p<0.05$).

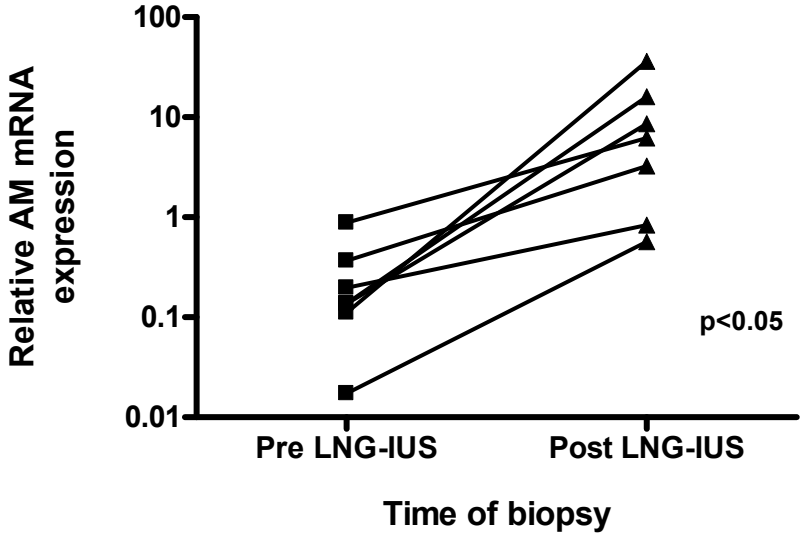


Figure 45. **The effect of a levonorgestrel releasing intra uterine system (LNG-IUS) on adrenomedullin (AM) mRNA expression**. AM mRNA in proliferative, early- and mid-secretory endometrium prior to IUS insertion was compared to paired samples collected 3-6 months after IUS insertion. Note logarithmic scale on y-axis.

4.3.3 Vascular endothelial growth factor (VEGF)

4.3.3.1 Regulation of VEGF in endometrial epithelial cells by hypoxia and prostaglandins E_2 and $F_{2\alpha}$

An endometrial epithelial cell line stably transfected with either the PGE_2 receptor (EP2S cells) or the $PGF_{2\alpha}$ receptor (FPS cells) was used to investigate the role of prostaglandins and hypoxia on VEGF expression. Treatment of EP2S cells with 100nM PGE_2 for 24h did not increase VEGF mRNA over vehicle treatment (Figure 50A). However, an increase in VEGF secreted protein was observed with this treatment ($p<0.01$) (Figure 46B). Incubation of these cells in hypoxia (0.5% O_2) for 24h showed a non-significant increase in VEGF mRNA (Figure 50A), with significant increases in secreted protein levels over vehicle treated cells ($p<0.001$) (Figure 46B). Maximal increases in VEGF mRNA and protein levels was observed when EP2S cells were co-treated with PGE_2 and hypoxic conditions ($p<0.05$, $p<0.001$) (Figure 46A,B).

FPS cells treated with 100nM $PGF_{2\alpha}$ or hypoxia showed a non-significant increase in VEGF mRNA, this increase reached significance when both factors were present simultaneously ($p<0.05$) (Figure 46C). VEGF secreted protein levels were significantly increased in the culture supernatants from FPS cells treated for 24h with $PGF_{2\alpha}$, hypoxia or both treatments together ($p<0.001$) (Figure 46D).

4.3.3.2 Regulation of VEGF levels in primary human endometrial stromal (HES) cells

HES cells treated with 100nM PGE_2 in normoxia or hypoxia showed a non-significant increase in VEGF mRNA expression over vehicle treated cells in normoxia (Figure 47A). There was a significant increase in VEGF secreted protein in culture supernatants from HES cells treated with PGE_2 in hypoxia for 24h ($p<0.05$) (Figure 47B). HES cells treated with 100nM $PGF_{2\alpha}$ in normoxia did not show increased VEGF secreted protein levels versus vehicle treated cells (Figure 47C). However, HES cells incubated in hypoxic conditions, with and without $PGF_{2\alpha,}$ had significantly elevated VEGF mRNA levels after 24h culture ($p<0.01$) (Figure 47C). Secreted VEGF protein levels were significantly elevated in the culture supernatant

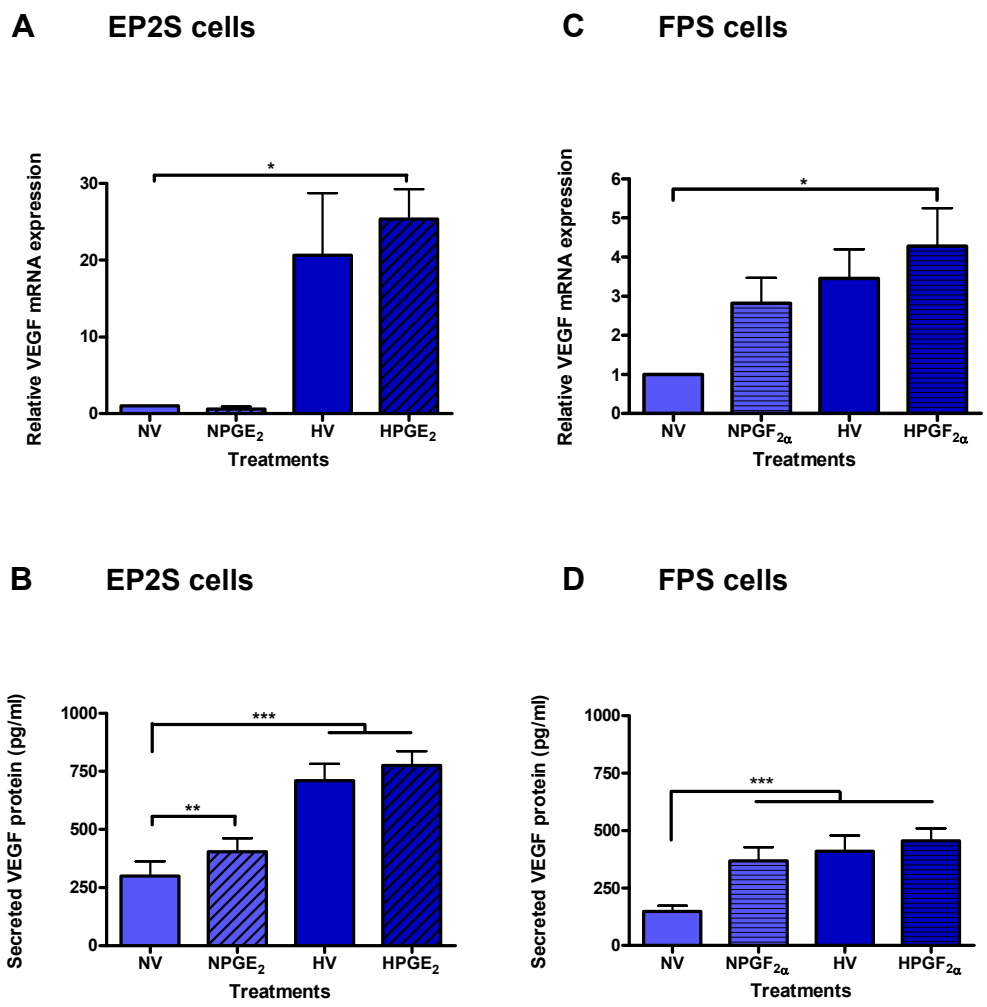


Figure 46. **The expression of vascular endothelial growth factor (VEGF) in endometrial epithelial cells treated with prostaglandins and hypoxia. (A)** VEGF mRNA in EP2S cells measured by Q-RT-PCR after 24h treatment with vehicle, 100nM prostaglandin E_2 (PGE_2), hypoxic conditions or both PGE_2 and hypoxia (n=3). **(B)** VEGF secreted protein levels measured by ELISA in the culture supernatants from these EP2S cells (n=3). **(C)** VEGF mRNA in FPS cells after 24h treatment with vehicle, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), hypoxic conditions or both $PGF_{2\alpha}$ and hypoxia (n=3). **(D)** VEGF secreted protein levels measured by ELISA in the culture supernatants from these FPS cells (n=3). N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle. (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

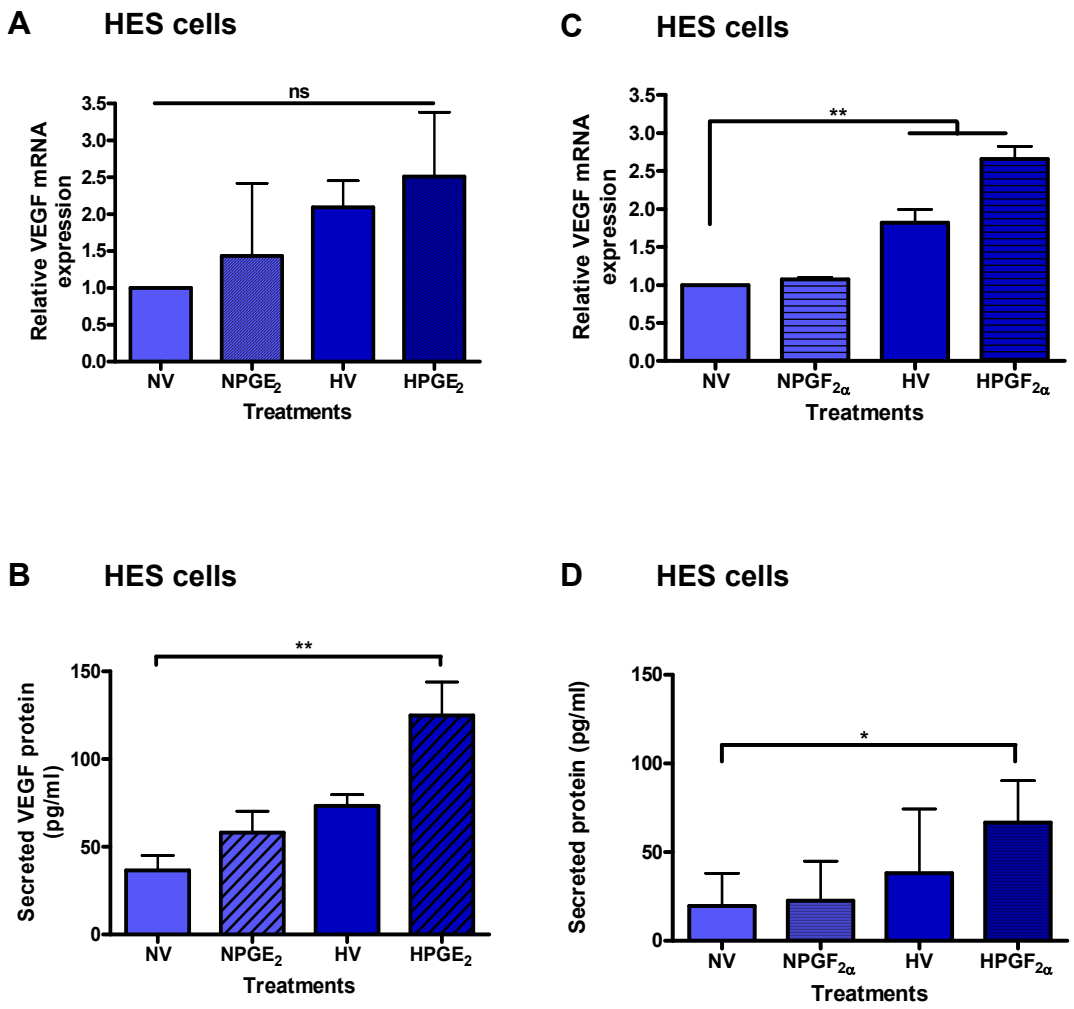


Figure 47. **The expression of vascular endothelial growth factor (VEGF) in primary human endometrial stromal (HES) cells treated with prostaglandins and hypoxia. (A)** VEGF mRNA in HES cells measured by Q-RT-PCR after 24h treatment with vehicle, 100nM prostaglandin E_2 (PGE_2), hypoxic conditions or both PGE_2 and hypoxia (n=3). **(B)** VEGF secreted protein levels measured by ELISA in the culture supernatants from these PGE_2/hypoxia treated HES cells (n=3). **(C)** VEGF mRNA in HES cells after 24h treatment with vehicle, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), hypoxic conditions or both $PGF_{2\alpha}$ and hypoxia (n=3). **(D)** VEGF secreted protein levels measured by ELISA in the culture supernatants from these $PGF_{2\alpha}$/hypoxia treated HES cells (n=3). N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle. (ns: non-significant, *$p<0.05$, **$p<0.01$).

from HES cells incubated for 24h with $PGF_{2\alpha}$ in hypoxic conditions versus vehicle treated cells in normoxia ($p<0.05$) (Figure 47D).

4.3.3.3 Regulation of VEGF levels in human endometrial tissue explants

Proliferative endometrial explants treated with 100nM PGE_2 or $PGF_{2\alpha}$ in normoxia or hypoxia showed no significant changes in VEGF mRNA (Figure 48A,B). Although changes were not significant, secretory tissue treated with prostaglandins and/or hypoxia showed a trend towards increased VEGF mRNA (Figure 48C,D). These differences between proliferative and secretory endometrium, suggest that prior progesterone exposure may influence the ability of tissue to respond to hypoxia and prostaglandin treatment.

4.3.3.4 The effect of *in vitro* progesterone, and its withdrawal, on VEGF expression

To test the hypothesis that prior progesterone exposure is necessary to increase endometrial VEGF expression, an *in vitro* model of progesterone withdrawal was utilised. Proliferative endometrial biopsies were divided into eight equal explants and pre-treated for 24h with synthetic progesterone (MPA). Explants were then co-treated with MPA plus (i) vehicle, (ii) indomethacin, a cyclo-oxygenase inhibitor, (iii) a progesterone receptor antagonist (RU486) or (iv) RU486 and indomethacin for 48h in either normoxic or hypoxic conditions. Tissue explants subjected to *in vitro* progesterone withdrawal in normoxia did not show significant up-regulation of VEGF mRNA (Figure 49A). However, *in vivo* progesterone withdrawal is followed by an episode of hypoxia as a result of spiral arteriole vasoconstriction. When progesterone antagonism was performed *in vitro* in hypoxic conditions to mimic the *in vivo* scenario more accurately, there was a significant increase in VEGF mRNA (Figure 49A). This increase was abrogated by co-treatment with indomethacin, suggesting that hypoxia and prostaglandin production are necessary for a significant increase in endometrial VEGF expression following progesterone withdrawal.

As progestins and RU486 may have off target effects, further endometrial explants were incubated with natural progesterone before withdrawal or maintenance of progesterone exposure. These experiments yielded similar results, with significantly

increased VEGF mRNA when explants were exposed to progesterone withdrawal in hypoxic conditions (Figure 49B).

4.3.3.5 The effect of local delivery of levonorgestrel on endometrial VEGF expression

To further examine the effect of progesterone exposure on VEGF expression, paired endometrial biopsies collected from the same woman before and 3-6 months after levonorgestrel-releasing intrauterine system (LNG-IUS) insertion (n=7) were examined. There was a significant up-regulation of VEGF mRNA in endometrium collected after LNG-IUS insertion when compared to pre-insertion biopsies taken in the proliferative and early-mid secretory phase ($p<0.05$) (Figure 50).

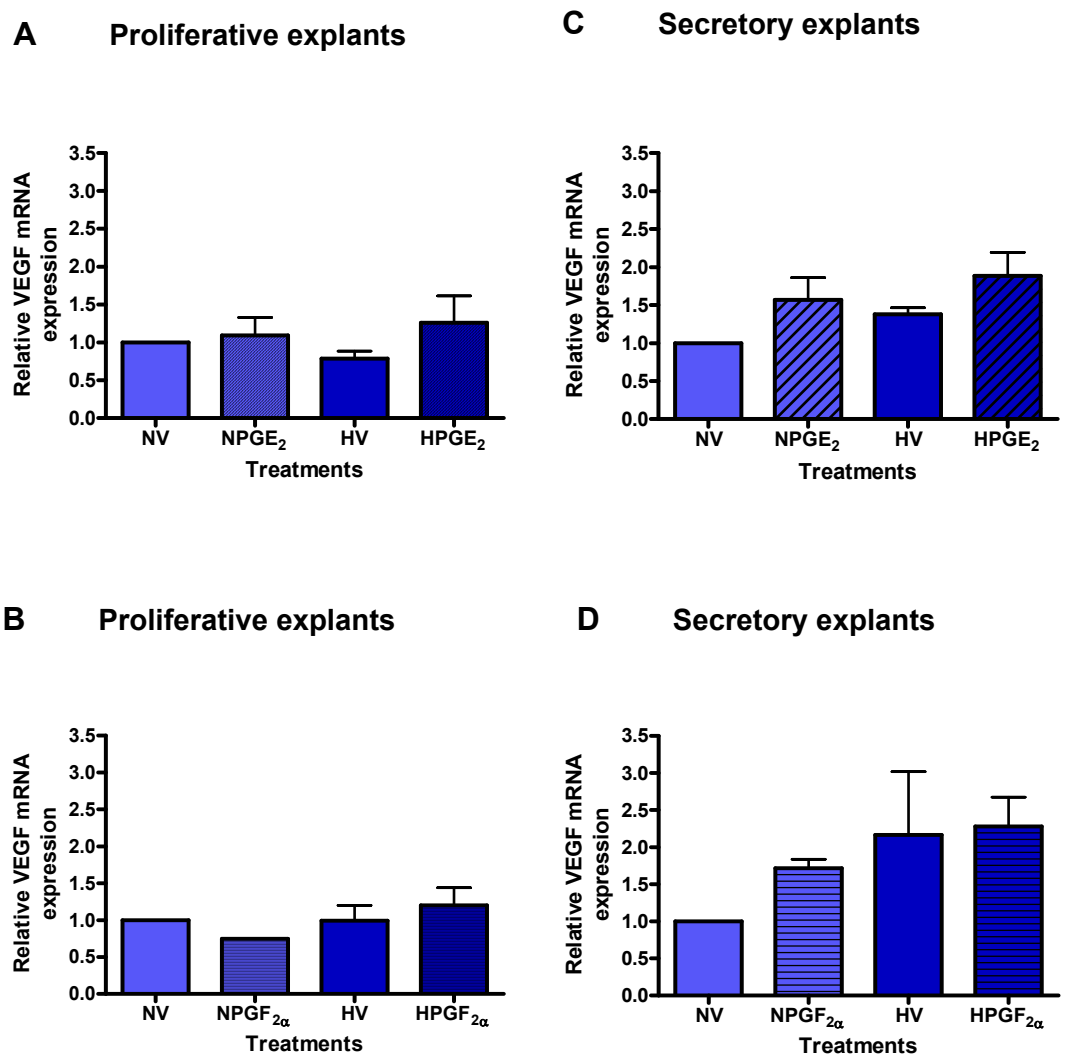
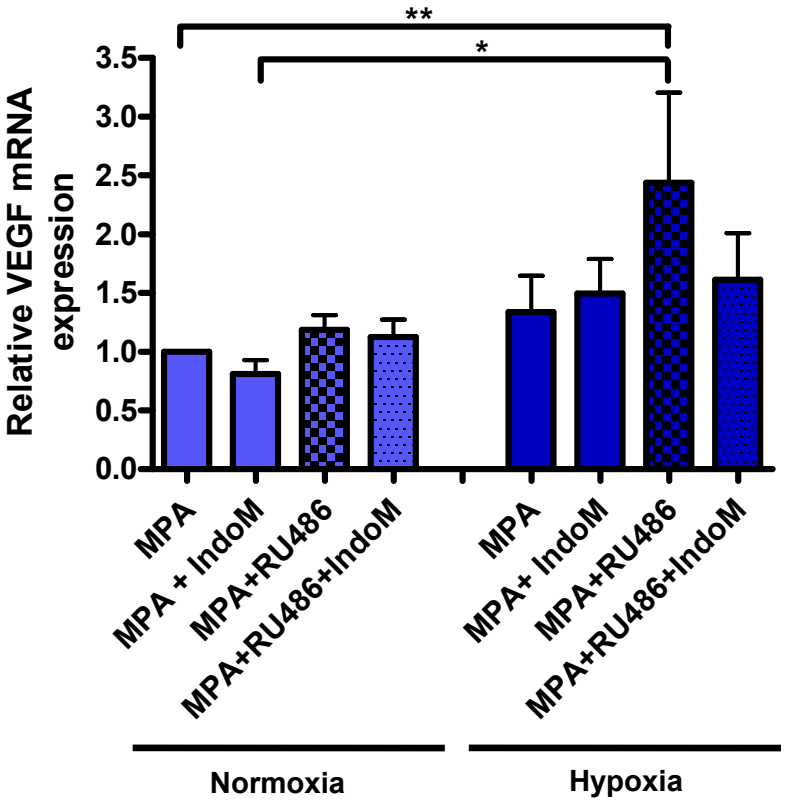


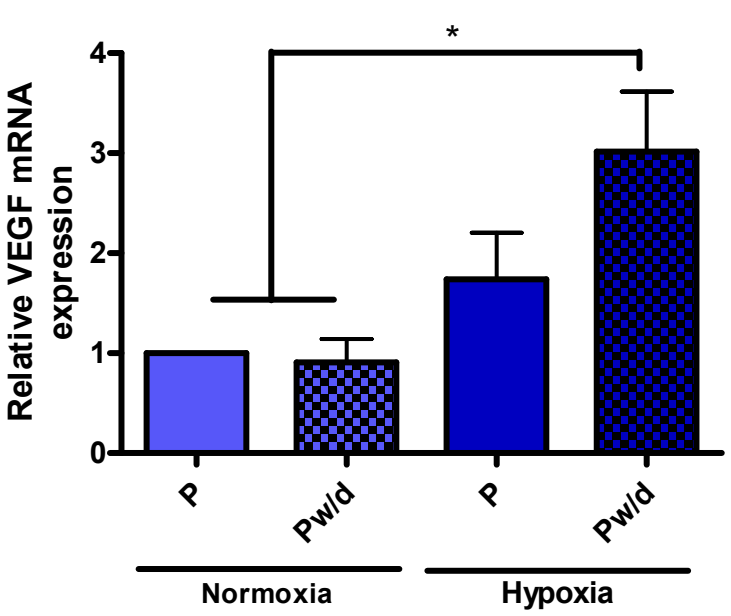
Figure 48. **The effect of prostaglandins $E_2/F_{2\alpha}$ and hypoxia on VEGF expression in endometrial explants.** VEGF mRNA in endometrial explants from the proliferative phase treated with **(A)** vehicle, 100nM PGE_2, hypoxia and both PGE_2 and hypoxia (n=3) or **(B)** vehicle, 100nM $PGF_{2\alpha}$, hypoxia or both hypoxia and $PGF_{2\alpha}$ (n=3) for 24h. **(C)** VEGF mRNA in secretory endometrial explants treated with vehicle, 100nM PGE_2, hypoxia and both PGE_2 (n=4). **(D)** VEGF mRNA in secretory endometrial explants treated with vehicle, 100nM $PGF_{2\alpha}$, hypoxia or both hypoxia and $PGF_{2\alpha}$ (n=3) for 24h. N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle.

Figure 49. **The effect of *in vitro* progesterone antagonism and withdrawal on endometrial VEGF expression. (A)** Proliferative phase explants (n=4) were pre-treated with1μM medroxyprogesterone acetate (MPA) and then (i) maintained in 1μM MPA, (ii) treated with MPA and indomethacin (MPA+IndoM), (iii) co-treated with 1μM MPA and 1μM RU486, a progesterone receptor antagonist (MPA+RU486) or (iv) co-treated with 1μM MPA, 1μM RU486 and 8.4μM indomethacin, a COX enzyme inhibitor (MPA+RU486+IndoM). Identical treatments were incubated in either normoxia (21% O_2) or hypoxia (0.5% O_2). **(B)** Four further endometrial biopsies were pre-treated with progesterone (1μM) before maintenance in progesterone (P) or incubation with control media to induce progesterone withdrawal (Pw/d) in normoxia and hypoxia. (*$p<0.05$, **$p<0.01$).

A


**
*
Relative VEGF mRNA expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MPA
MPA + IndoM
MPA+RU486
MPA+RU486+IndoM
MPA
MPA+ IndoM
MPA+RU486
MPA+RU486+IndoM
Normoxia
Hypoxia

B


*
Relative VEGF mRNA expression
4
3
2
1
0
P
Pw/d
P
Pw/d
Normoxia
Hypoxia

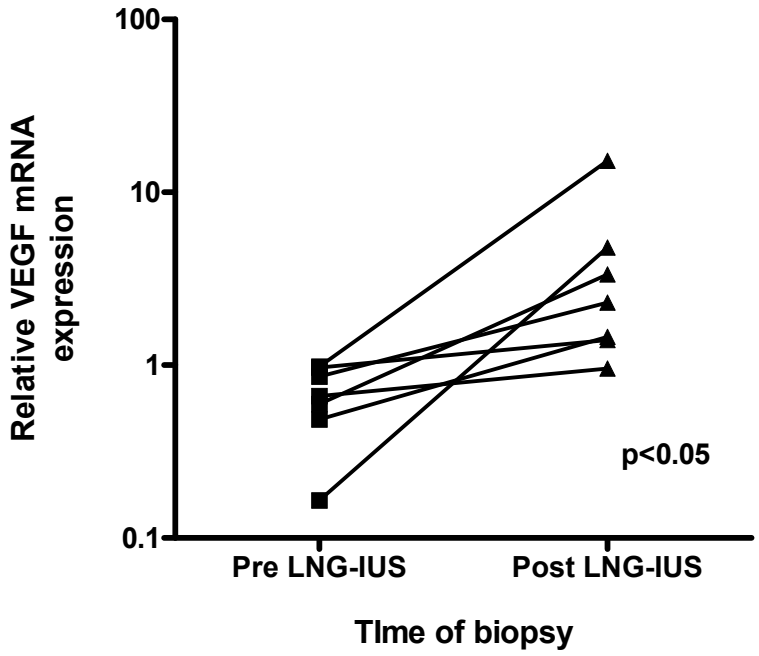


Figure 50. **The effect of a levonorgestrel releasing intra uterine system (LNG-IUS) on VEGF mRNA.** Insertion of the LNG-IUS, with consequent local "P-deprivation" analogous to local "P-withdrawal", resulted in a significant increase in VEGF mRNA, note logarithmic scale on y-axis.

4.3.4 Connective tissue growth factor (CTGF)

4.3.4.1 The effect of PGE_2 and hypoxia on CTGF expression in EP2S cells

A time course experiment in EP2S cells revealed a significant increase in CTGF mRNA on treatment with 100nM PGE_2 compared to vehicle at 4 and 8h ($p<0.01$) (Figure 51A). Hypoxic conditions (0.5% O_2) also significantly increased CTGF mRNA in these cells at 2h versus normoxic controls ($p<0.001$) (Figure 51B). Interestingly, in prolonged hypoxic conditions this up-regulation did not take place. When cells were treated with both PGE_2 and hypoxia for 2h there was a synergistic increase in CTGF mRNA, that was significantly greater than that seen with either treatment alone ($p<0.05$) (Figure 52A). As maximal CTGF mRNA occurred between 2 and 4h, the amount of CTGF protein secreted into culture supernatants was assessed by ELISA at a 6h time point. CTGF secreted protein was significantly increased over vehicle treated cells when EP2S cells were co-treated with PGE_2 and hypoxia ($p<0.05$) (Figure 52B).

4.3.4.2 The effect of $PGF_{2\alpha}$ and hypoxia on CTGF expression in FPS cells

Treatment of FPS cells with 100nM $PGF_{2\alpha}$ for 6 and 24h resulted in significant up-regulation of CTGF mRNA versus vehicle treated cells ($p<0.05$ and $p<0.001$ respectively) (Figure 53A). As in EP2S cells, hypoxic conditions did not significantly increase CTGF mRNA at 6 or 24h in FPS cells (Figure 53A). Secreted protein levels in culture supernatants at 6 and 24h demonstrated significant increases in CTGF protein over controls after treatment with $PGF_{2\alpha}$ ($p<0.001$) (Figure 53B).

4.3.4.3 The effect of prostaglandins and hypoxia on CTGF expression in endometrial tissue explants

As 100nM $PGF_{2\alpha}$ had the most marked effect on CTGF expression in endometrial cells, endometrial tissue explants were cultured with this prostaglandin for 24h. This did not result in a significant increase in CTGF expression, regardless of the cycle stage of the tissue (Figure 54A,B). In contrast, hypoxic conditions did produce a significant increase in CTGF mRNA over controls in both proliferative ($p<0.05$) and secretory ($p<0.001$) explants (Figure 54A,B). Due to the limitations of

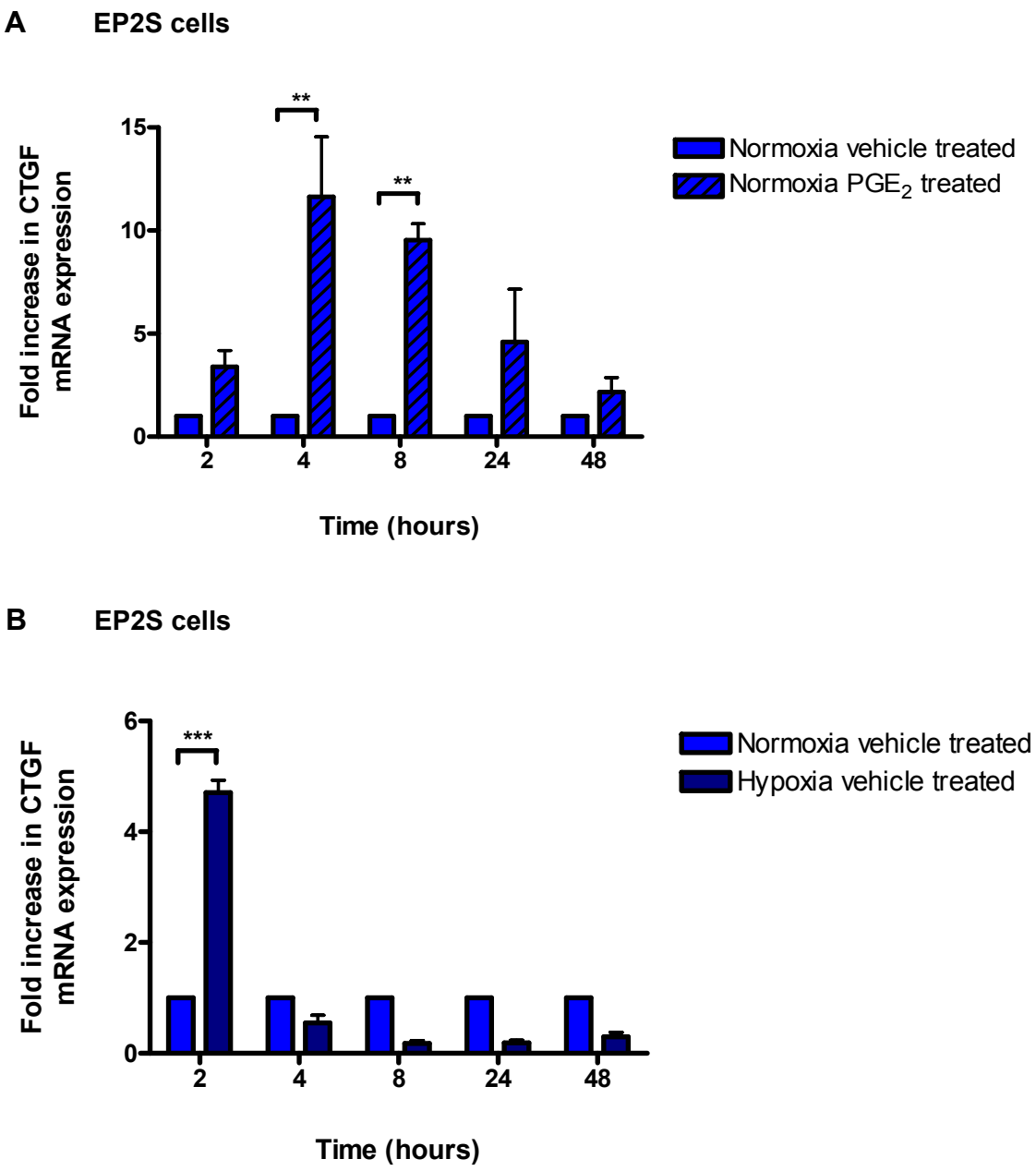


Figure 51. **Connective tissue growth factor mRNA in endometrial epithelial cells (Ishikawa EP2S cells) treated with prostaglandin E_2 and hypoxia.** (A) CTGF mRNA measured by Q-RT-PCR in EP2S cells treated with vehicle or 100nM PGE_2 for up to 48h in normoxic conditions (21% O_2) (n=3). (B) EP2S cells cultured in normoxic (21% O_2) and hypoxic (0.5% O_2) conditions for up to 48h (n=3). (**p<0.01, ***p<0.001).

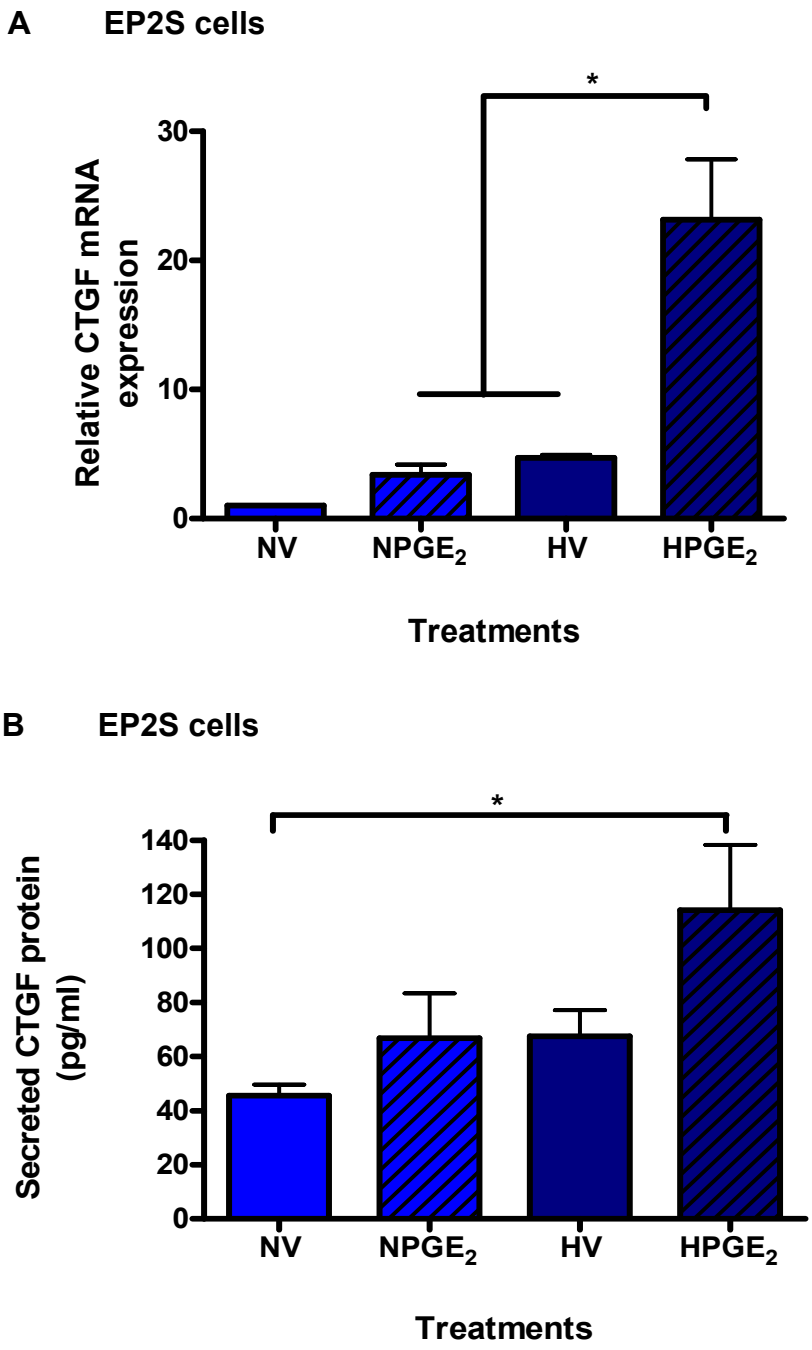


Figure 52. The impact of simultaneous treatment of EP2S cells with 100nM prostaglandin E_2 and hypoxia on **(A)** connective tissue growth factor (CTGF) mRNA expression measured by Q-RT-PCR at 2h (n=3) and **(B)** secreted CTGF protein levels measured by ELISA in EP2S culture supernatants at 6h (n=3). N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle, PGE_2: prostaglandin E_2. (*$p<0.05$).

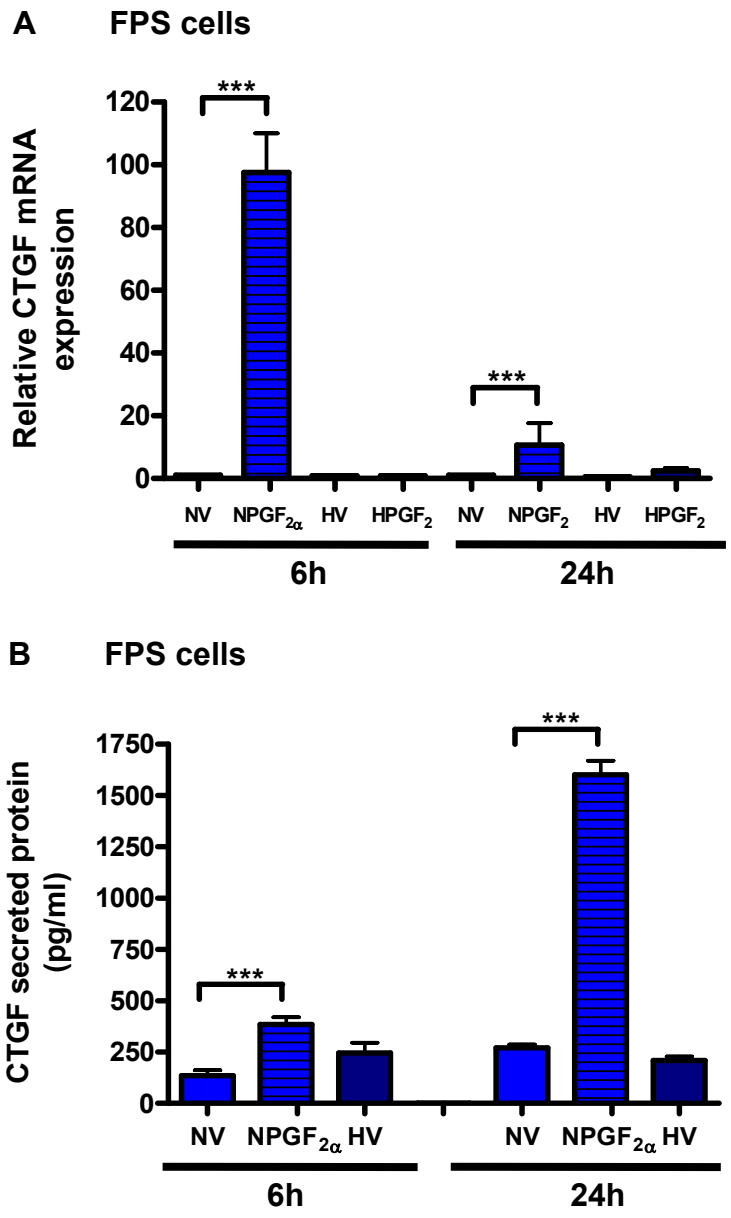


Figure 53. **The impact of prostaglandin $F_{2\alpha}$ and hypoxia on connective tissue growth factor (CTGF) mRNA in endometrial epithelial cells (Ishikawa FPS cells).** (A) CTGF mRNA measured by Q-RT-PCR in FPS cells treated with vehicle, 100nM $PGF_{2\alpha}$, hypoxia or $PGF_{2\alpha}$ and hypoxia for 6h and 24h (n=3). (B) CTGF secreted protein levels measured by ELISA in culture supernatants from FPS cells treated for 24h with vehicle, $PGF_{2\alpha}$ or hypoxia (n=3). N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle, $PGF_{2\alpha}$: 100nM prostaglandin $F_{2\alpha}$ (***$p<0.001$).

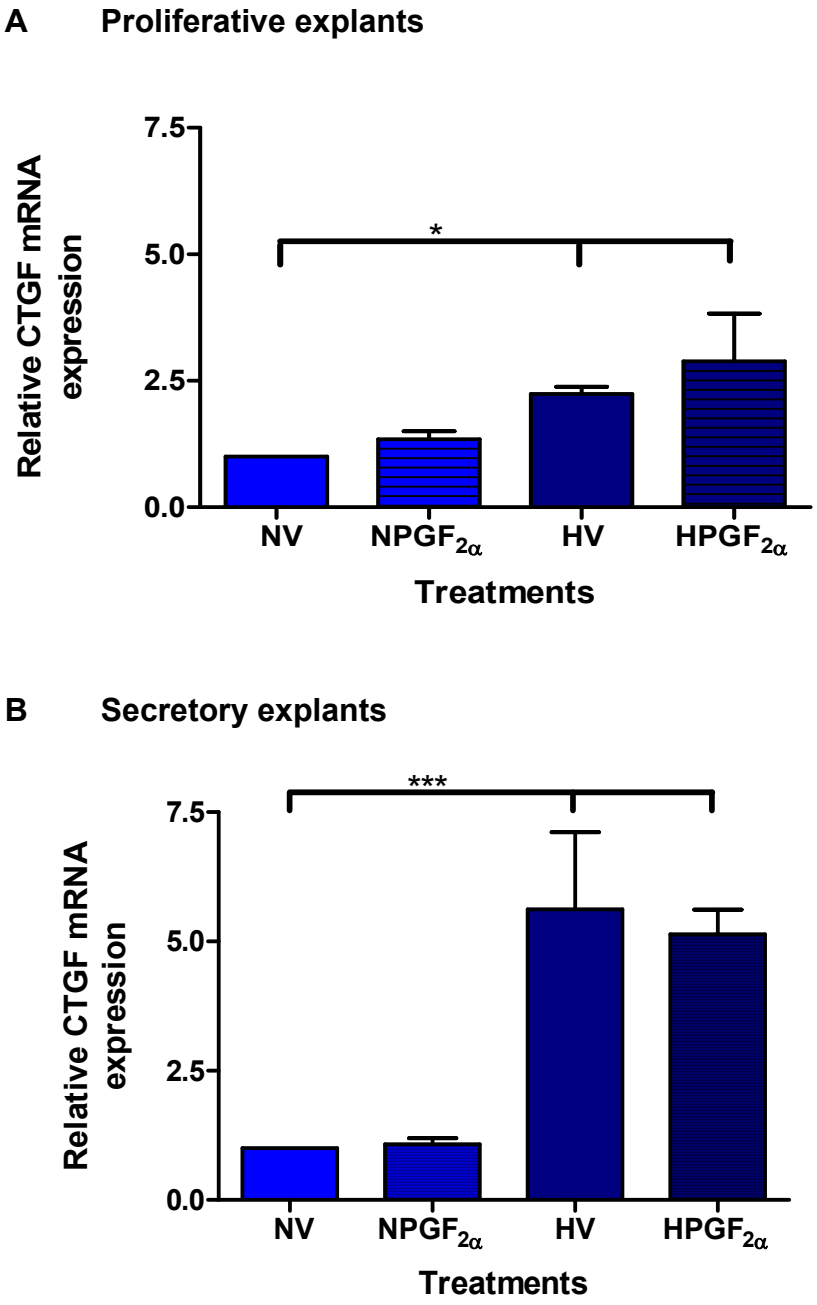


Figure 54. **The effect of prostaglandin $F_{2\alpha}$ and hypoxia on CTGF expression in endometrial tissue explants. (A)** Endometrial explants from the proliferative phase treated with vehicle, 100nM $PGF_{2\alpha}$, hypoxia or $PGF_{2\alpha}$ and hypoxia for 24h (n=3). **(B)** Endometrial tissue explants from the secretory phase treated in an identical manner (n=3). N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle, $PGF_{2\alpha}$: 100nM prostaglandin $F_{2\alpha}$. (*$p<0.05$, ***$p<0.001$).

endometrial tissue availability, the effects of PGE_2 on endometrial tissue explants at 2h could not be assessed.

4.3.4.4 The effect of *in vitro* progesterone and its antagonism on CTGF expression

Endometrial explants subjected to an *in vitro* model of progesterone withdrawal showed no significant changes in CTGF mRNA (Figure 55). These explants were maintained in progesterone before co-treatment with RU486 in normoxic and hypoxic conditions to mimic progesterone withdrawal. Antagonism of progesterone in normoxic conditions revealed a non-significant increase in CTGF expression, which was abrogated when COX-2 was inhibited with indomethacin. Interestingly, in contrast to other repair factors examined, hypoxic conditions significantly reduced CTGF mRNA when compared to normoxic explants, regardless of co-treatments.

4.3.4.5 The effect of LNG-IUS insertion on endometrial CTGF expression

To further examine the effect of progesterone exposure on CTGF expression, paired endometrial biopsies collected from the same woman before and 3-6 months after levonorgestrel-releasing intrauterine system (LNG-IUS) insertion (n=7) were examined as an *in vivo* model of progesterone "deprivation". In contrast to the other repair factors examined, there was no significant up-regulation of CTGF mRNA in endometrium collected after LNG-IUS insertion when compared to pre-insertion biopsies (p=0.0781) (Figure 56).

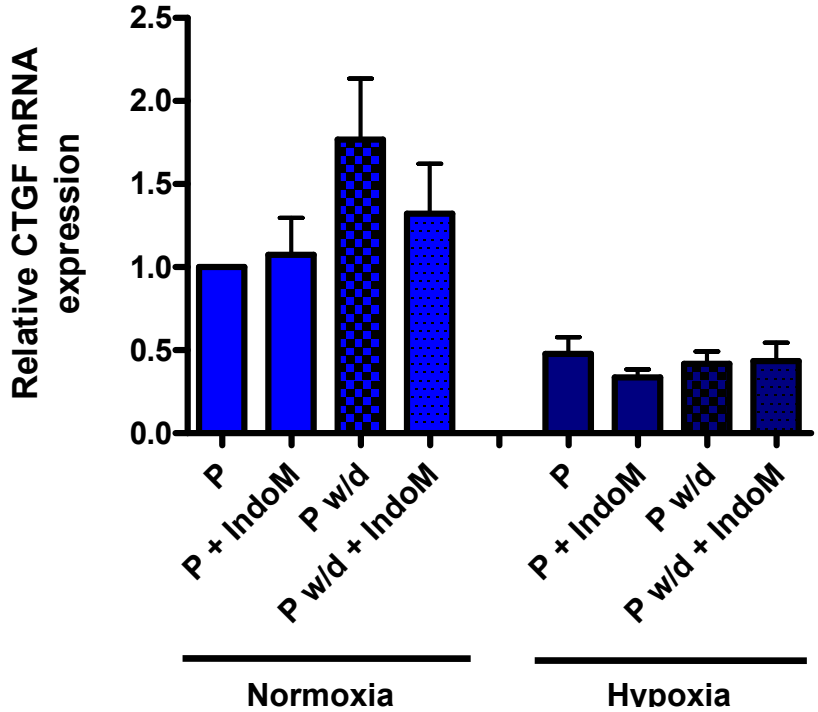


Figure 55. **The effect of *in vitro* progesterone antagonism on endometrial CTGF expression.** Proliferative phase explants (n=4) were pre-treated with1μM medroxyprogesterone acetate (MPA) and then (i) maintained in 1μM MPA (P), (ii) co-treated with 1μM MPA and 1μM RU486, a progesterone receptor antagonist (Pw/d) or (iii) co-treated with 1μM MPA, 1μM RU486 and 8.4μM indomethacin, a COX enzyme inhibitor (Pw/d + IndoM). Identical treatments were incubated in either normoxia (21% O_2) or hypoxia (0.5% O_2).

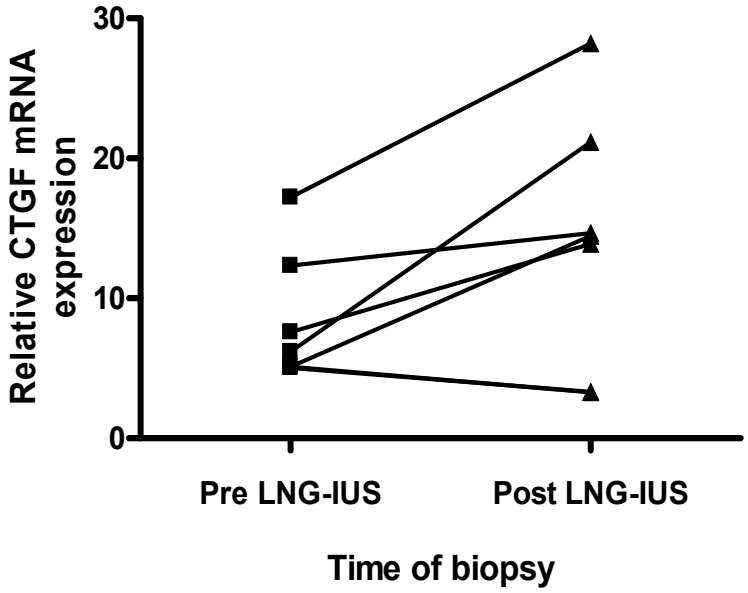


Figure 56. **The effect of a levonorgestrel releasing intra uterine system (LNG-IUS) on CTGF mRNA.** Insertion of the levonorgestrel releasing intra uterine system (IUS), with consequent local "P-deprivation" analogous to local "P-withdrawal", resulted in a significant increase in CTGF mRNA.

4.3.5 Prostaglandin receptor expression in endometrial explants

To assess the impact of hypoxia and prostaglandin treatment on prostaglandin receptor expression, EP2 and FP mRNA was measured in proliferative and secretory endometrial explants after 24h culture. There were no significant changes in EP2 or FP mRNA after treatment with 100nM PGE_2, 100nM $PGF_{2\alpha}$, hypoxia (0.5% O_2) or prostaglandins and hypoxia (Figure 57A-D).

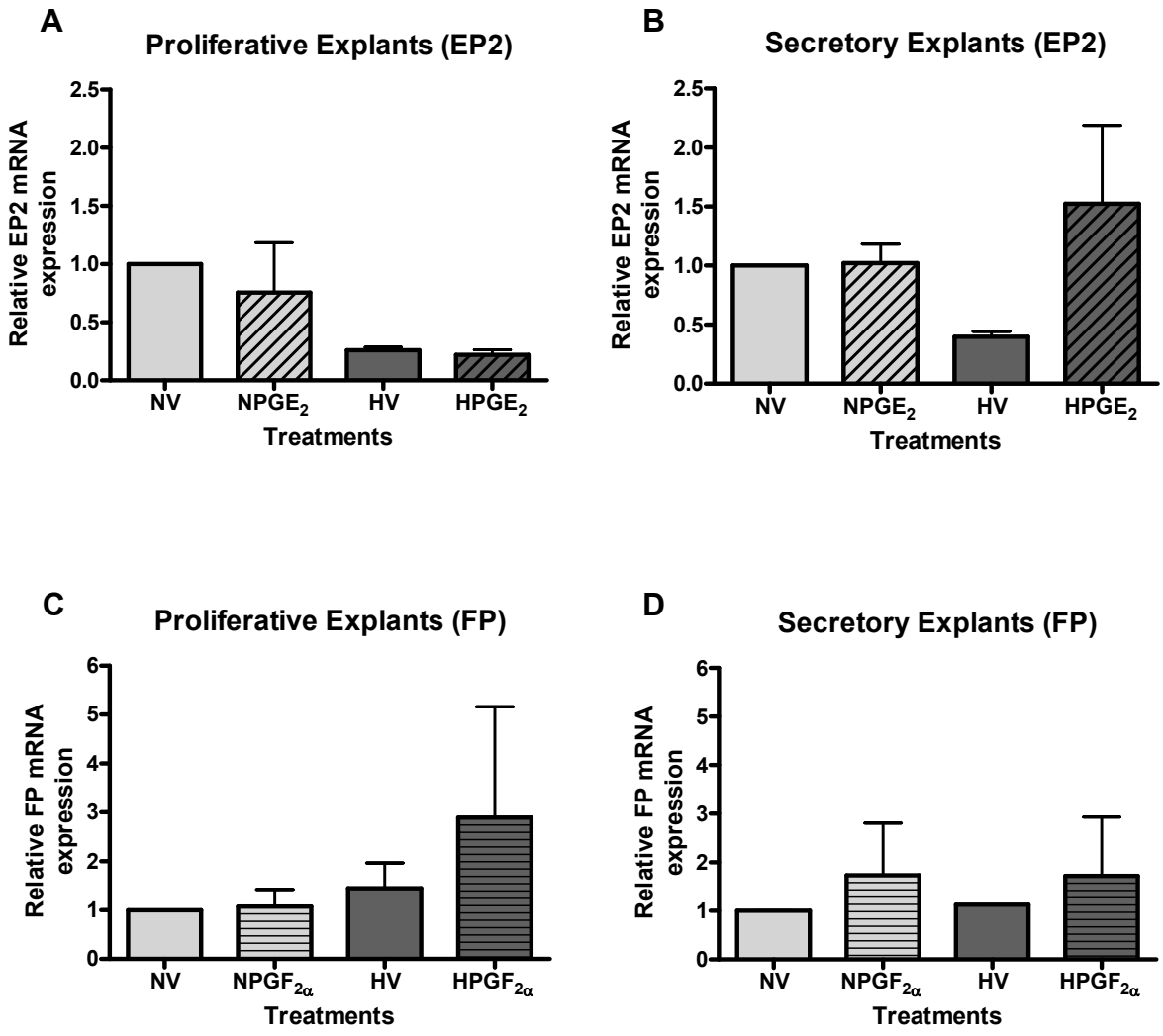


Figure 57. **The effect of prostaglandins and hypoxia on endometrial explant prostaglandin receptor expression**. **(A)** EP2 mRNA in endometrial explants from the proliferative phase treated with vehicle, 100nM PGE_2, hypoxia or PGE_2 and hypoxia for 24h (n=3). **(B)** EP2 mRNA in secretory tissue explants treated in an identical manner (n=3). **(C)** FPS mRNA in endometrial explants from the proliferative phase treated with vehicle, 100nM prostaglandin $F_{2\alpha}$, hypoxia or $PGF_{2\alpha}$ and hypoxia for 24h (n=3). **(D)** FPS mRNA in secretory phase explants treated identically (n=3). N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: ethanol vehicle, PGE_2: 100nM prostaglandin E_2, $PGF_{2\alpha}$: 100nM prostaglandin $F_{2\alpha}$.

4.4 Discussion

The *in vitro, ex vivo* and *in vivo* experiments described in this chapter reveal that endometrial repair factors are increased after treatment with prostaglandins and hypoxia. Exposure to progesterone and its subsequent withdrawal appears essential before prostaglandins and hypoxia can initiate expression of IL-8, AM and VEGF for endometrial repair. Although data in the results section of this chapter are presented by gene, this discussion focuses on the application of these individual results to overall endometrial processes and their regulation.

4.4.1 The role of oestrogen in endometrial repair

As oestradiol is the dominant hormone in the proliferative phase, it was traditionally thought to regulate endometrial repair and remodelling. However, there is mounting evidence that oestrogen is not required for the initial stages of repair. Firstly, electron microscopy and hysteroscopy studies of the human endometrium have revealed that endometrial repair commences during active bleeding, when oestrogen levels remain low (Ludwig and Spornitz, 1991, Garry et al., 2009). In addition, early human endometrial repair takes place in the absence of ovarian hormones, e.g. after ovariectomy.

Ovariectomised animal models provide further support for the oestrogen independent nature of endometrial repair. In the mouse model of simulated menstruation (Kaitu'u-Lino et al., 2007a), ovariectomised mice were exposed to cyclical E_2 and progesterone. After initiation of decidualisation, progesterone implants were withdrawn to simulate menstruation. One group of mice were given subcutaneous injections of 17β oestradiol from the time of progesterone withdrawal. The other group were given injections of letrozole, a third generation aromatase inhibitor, in place of E_2. In addition, the oestrogen deplete group were maintained on a soy free diet to prevent extra ovarian oestrogen exposure. No significant difference in the rate of endometrial repair was observed between the two groups.

Studies in the ovariectomised macaque model concur with findings in the mouse. On examination of the endometrial repair factor VEGF, there were three peaks of mRNA

expression; in the surface epithelium during the simulated early proliferative phase, in the stroma during the mid proliferative phase and in the glands during the late secretory phase. Comparison of hormone deprived and E_2 exposed animals revealed that E_2 was not essential for the early proliferative phase peak but was necessary for the increase in stromal VEGF during the mid-proliferative phase. Authors concluded that VEGF played a dual role in the endometrium. Firstly, to repair vessels damaged at menstruation, which occurs during the early proliferative phase. This does not require the presence of oestrogen. Secondly, that VEGF contributes to endometrial remodelling during the mid-proliferative stage of the cycle; an event with an absolute requirement for oestrogen (Nayak and Brenner, 2002). The same ovariectomised macaque model revealed that fibronectin, a large glycoprotein that interacts with specific integrins to enhance cell adhesion and migration during wound repair (Kim et al., 1992), is also up-regulated by progesterone withdrawal (Cao et al., 2007). Again, the 50 fold increase in fibronectin expression observed in menstrual versus secretory phase endometrium, was independent of oestrogen.

4.4.2 Progesterone withdrawal in the regulation of endometrial repair

As initial endometrial repair appears to occur in the absence of E_2, it was hypothesized that progesterone withdrawal was the trigger for endometrial repair factor expression. Herein, we have demonstrated that prostaglandins and hypoxia independently up-regulate IL-8 and AM mRNA in endometrial explants that have had prior progesterone exposure. To test this hypothesis, proliferative phase endometrial explants were subjected to a previously described *ex vivo* model of progesterone antagonism (Critchley et al., 2003), to mimic the progesterone withdrawal that occurs *in vivo* during the late secretory phase. When explants were co-treated with progesterone and a progesterone receptor antagonist, there was no significant increase in IL-8, VEGF, AM or CTGF mRNA when compared to explants maintained in progesterone. However, when progesterone is withdrawn *in vivo* the spiral arterioles constrict, resulting in a hypoxic episode that was not accounted for in this *ex vivo* model. Further explants from the same biopsies, therefore, were treated in an identical manner but placed in a hypoxic chamber. Progesterone withdrawal in hypoxic conditions significantly increased IL-8, VEGF

and AM expression when compared to explants treated in normoxic conditions. Addition of the COX inhibitor indomethacin abrogated this up-regulation. These data suggest that progesterone withdrawal does up-regulate endometrial repair factors but only in the presence of hypoxia and prostaglandins. An alternative explanation for the findings in this *ex vivo* progesterone antagonism model may be that increased endometrial repair factor expression is secondary to inhibition of the glucocorticoid receptor (GR). Mifepristone (RU486) is not only a progesterone receptor antagonist but also a GR antagonist. To further explore this, tissue explants were exposed to natural progesterone, followed by its withdrawal. In this model, VEGF mRNA was only significantly increased when progesterone was withdrawn in hypoxic conditions. These results are consistent with findings following progesterone antagonism. Further analysis of AM, IL-8 and CTGF expression in the progesterone withdrawal model is necessary. Interestingly, CTGF mRNA was significantly decreased by hypoxic conditions in the progesterone antagonism model. Data from cell experiments revealed that hypoxic induction of CTGF was rapid, occurring by 2h. Therefore, exposure of endometrial tissue to prolonged hypoxia may mask the induction of CTGF. Unfortunately, due to limited tissue availability, these experiments could not be repeated with shorter incubation times.

To further examine the contribution of progesterone to endometrial repair, endometrial biopsies were collected from women before and 3-6 months after LNG-IUS insertion for treatment of HMB. LNG-IUS insertion has been shown to dramatically down-regulate endometrial progesterone receptors, hence provides an *in vivo* model of "progesterone deficiency" (Critchley et al., 1998) (Critchley et al., 2006a). In addition, progestogen exposure has been shown to reduce endometrial perfusion and profoundly decrease vasomotion (Hickey et al., 2006). This may lead to endometrial hypoxia. Endometrial samples collected 3-6 months after LNG-IUS insertion showed significant increases in IL-8, AM and VEGF expression. As the normal endometrial architecture is maintained in this *in vivo* model, these findings support the role of progesterone withdrawal and downstream hypoxia in the up-regulation of endometrial repair factors. Alternatively, the increased expression of these endometrial factors after local exposure to levonorgestrel may be due to

induction of decidualisation. Angiogenic factors appear essential for implantation and early pregnancy and have been identified in first trimester decidua tissue (Marinoni et al., 2004, Wei et al., 2004). The LNG-IUS is well established as a treatment for HMB and its stimulation of repair factor production may contribute to its efficacy.

The elevation in IL-8 mRNA seen in this LNG-IUS *in vivo* model of progesterone "deprivation" is comparable to the findings in endometrial samples from women taken 48h after withdrawal of vaginal progesterone administration, versus endometrium from controls during the normal mid-secretory phase (Critchley et al., 1999). This study detected significantly increased IL-8 and COX-2 mRNA 48h after progesterone withdrawal. However, findings in this chapter are in contrast to microarray analysis of endometrium taken from women 24h after a single dose of mifepristone, to simulate progesterone withdrawal *in vivo* (Catalano et al., 2007). IL-8, AM or VEGF were not found to be differentially expressed between the treated group and normal controls in the mid-secretory phase. This discrepancy may be explained by the timing of the endometrial biopsy. If progesterone withdrawal stimulates COX-2 to induce synthesis of prostaglandins and a hypoxic episode, which in turn increase the expression of repair factors, it follows that the time from progesterone withdrawal to repair factor up-regulation is likely to exceed 24h. Sampling of endometrium pre- and 48h post-mifepristone administration may reveal significant increases in IL-8, AM and VEGF mRNA, similar to that seen in endometrium exposed to the LNG-IUS. Similarly, another study examined gene expression in mid- versus late-secretory endometrial biopsies as a model of physiologic progesterone withdrawal (Critchley et al., 2006c). Gene array technology failed to identify significant increases in IL-8, AM or VEGF mRNA between the two groups. Functional analysis of the genes that were differentially expressed between mid- and late-secretory endometrium identified bioprocesses such as haemostasis, steroid biosynthesis and prostaglandin metabolism. These processes are consistent with the early response to progesterone withdrawal and support the hypothesis that endometrial repair factors are induced greater than 48h after progesterone withdrawal.

4.4.3 The role of hypoxia in the regulation of endometrial repair factors

Although the contribution of hypoxia to the initiation of menstruation is currently controversial (Zhang and Salamonsen, 2002), the results described in this chapter support an important role for hypoxia in the initiation of endometrial repair, through IL-8, AM and VEGF induction. Evidence for the presence of a transient hypoxia in the uppermost endometrial zones during the peri-menstrual phase is discussed in Chapter 1.5.1. The hypoxic regulation of angiogenic factors is well described in pathological conditions, such as the hypoxic environment within a tumour (Sivridis et al., 2002, Carmeliet, 2005, Nikitenko et al., 2006b). Hypoxic regulation of IL-8 has been demonstrated in lung macrophages (Hirani et al., 2001) and may be associated with the onset of acute respiratory distress syndrome. Induction of AM expression by hypoxia has been seen in canine kidney cells (Nagata et al., 1999) and has also been identified in the placenta of women with pre-eclampsia (Gratton et al., 2003). The role of hypoxia in the regulation of angiogenic factors in physiological situations is less well documented. Previous studies have shown hypoxic induction of VEGF in isolated endometrial stromal and epithelial cells (Sharkey et al., 2000) and results herein support these findings. Of note, the response to hypoxia differed between EP2S and FPS cells. Reasons for this variation are unclear but may be secondary to the transfection process; therefore findings were confirmed with endometrial explant studies where possible. The hypoxic induction of IL-8, AM, VEGF and CTGF in an endometrial cell line, primary human endometrial cells and endometrial tissue, support a role for local hypoxia in endometrial repair.

4.4.4 The role of prostaglandins in the regulation of endometrial repair factors

Following the decline in progesterone levels during the late secretory phase, endometrial COX-2 mRNA is increased and subsequently stimulates prostaglandin synthesis (Critchley et al., 1999, Baird et al., 1996) (See Figure 3, Chapter 1). Examination of COX-2 deficient mice revealed multiple reproductive failures, including ovulation, fertilization, implantation and decidualisation (Langenbach et al., 1999a, Langenbach et al., 1999b, Dinchuk et al., 1995). COX-2 and subsequent production of PGE_2 has been demonstrated to have a role in the regulation of

endometrial cell survival and migration (Banu et al., 2008). Endometrial PGE synthase, PGE_2 and the EP2/EP4 receptors have all been detected in the human endometrium throughout the menstrual cycle (Milne et al., 2001). Endometrial cAMP generation in response to PGE_2 was significantly greater in proliferative versus early- to mid-secretory tissue. Similarly, the $PGF_{2\alpha}$ receptor (FP) has also been identified in the human endometrium, with significantly greater mRNA expression during the proliferative versus secretory phase (Milne and Jabbour, 2003). PGE_2 and $PGF_{2\alpha}$ are emerging as potential key players in the initiation of endometrial repair factors. Both of these prostaglandins have previously been shown to increase angiogenic factors in cancer cells (Sales et al., 2005, Sales et al., 2004, Fukuda et al., 2003, Keightley et al., 2010, Sales et al., 2009). The findings described in this chapter support and extend these studies, by describing increases in IL-8, AM and VEGF on exposure to PGE_2/ $PGF_{2\alpha}$ in normal human endometrial explants. Interestingly, $PGF_{2\alpha}$ significantly up-regulated endometrial AM expression but PGE_2 had no such effect, suggesting each prostaglandin has a specific endometrial action. Inflammatory cytokines, such as tumour necrosis factor alpha and interleukin 1β, have previously been shown to regulate AM expression (Hofbauer et al., 2002), but data presented in this chapter is the first report of AM regulation by $PGF_{2\alpha}$. Due to time and the constraints of tissue availability, the contribution of prostacyclin (PGI) was not examined herein. A previous study of endometrial tissue explants treated with 100nM iloprost (a PGI analogue) revealed significant increases in the expression of the angiogenic genes bFGF, Ang-1 and Ang-2 (Smith et al., 2006), suggesting it may also contribute to the induction of endometrial repair. Interestingly, the increases in endometrial repair factors described in this chapter were limited to endometrial tissue that had been exposed to progesterone. To determine if this effect was due to differences in prostaglandin receptor expression, EP2 and FP gene expression was examined in explants after 24h treatment. There were no significant differences in receptor expression after treatment with PGE_2, $PGF_{2\alpha}$ or hypoxia (Figure 57). These results suggest that the differences in repair gene expression were not a consequence of varied prostaglandin receptor expression. These findings are supported by the identification of EP2 and FP protein by immunohistochemistry in

both proliferative and secretory endometrial tissue (Milne and Jabbour, 2003, Milne et al., 2001).

4.4.5 Summary

The *in vitro, ex vivo* and *in vivo* models detailed in this chapter demonstrate that PGE_2, $PGF_{2\alpha}$ and hypoxic conditions have a role in the endometrial expression of the repair factors IL-8, AM, VEGF and CTGF. Tissue explant studies have revealed that exposure to progesterone, followed by its withdrawal, is necessary to allow prostaglandins and hypoxia to take effect. These experiments detail a potential mechanism for the initiation of repair factors in the perimenstrual human endometrium (Figure 58).

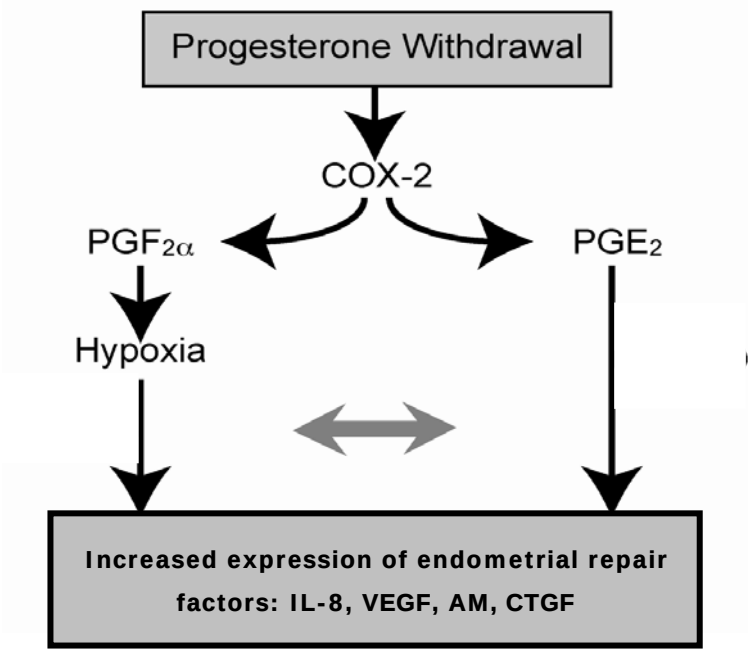


Figure 58. **Up-regulation of repair factors in the perimenstrual endometrium.** Elevated COX-2 following progesterone withdrawal induces synthesis of PGE_2 and PG $F_{2\alpha}$. PG $F_{2\alpha}$ is a potent vasoconstrictor and, alongside other vasoconstrictors, causes an episode of transient hypoxia in the superficial endometrial zones. Progesterone withdrawal, prostaglandins and hypoxic conditions are required to increase endometrial repair factors. Interaction between these pathways may lead to synergistic increases in repair factor mRNA expression.

5. The Role of Hypoxia Inducible Factor in Human Endometrial Repair

5.1 Introduction

As detailed in Chapter 4, hypoxic conditions and prostaglandins can significantly increase endometrial repair factors. These effects may be mediated via hypoxia inducible factor-1 (HIF-1), a heterodimeric transcription factor known to regulate the cellular response to hypoxia at other tissue sites. HIF was first identified in the kidney, where it was found to have a role in the regulation of erythropoietin transcription (Wang et al., 1995). Subsequently, over one hundred genes have been identified as transcriptional targets of HIF, reviewed in (Wiesener and Maxwell, 2003). These downstream targets include genes involved in glucose uptake and metabolism, extracellular matrix remodelling/digesting proteinases (Krishnamachary et al., 2003) and angiogenic/tissue repair genes such as vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), endothelin (ET), and angiopoietin 2 (Ang-2) (Semenza, 2000, Smith, 2001, Higgins et al., 2004).

The HIF complex is composed of an α and a β subunit. The α subunit is oxygen-regulated (Wang et al., 1995, Jiang et al., 1997), whereas the β subunit is constitutively expressed. When oxygen is abundant, the HIF-1α subunit is hydroxylated at two proline residues by a group of prolyl hydroxylases (PHDs). These enzymes utilize oxygen and the tricyclic acid cycle intermediate 2-oxyglutarate as substrates (Bruick and McKnight, 2001). Hydroxylation facilitates binding of the von Hippel-Lindau (VHL) protein and the VHL-HIF complex is rendered a target for the E3 ubiquitin-protein ligase, leading to proteasomal degradation (Jaakkola et al., 2001, Maxwell et al., 1999). During hypoxic conditions hydroxylation cannot take place and HIF-1α escapes proteasomal degradation. This allows dimerisation with HIF-1β to form an active transcription complex. In the ferret lung, it has been demonstrated that HIF-1α protein is stabilised in an oxygen concentration-dependent manner, with maximal expression after 4h of 0-1% oxygen ventilation (Yu et al., 1998). On reoxygenation, HIF-1α was rapidly degraded, with a half life of less than 1 minute. This tight regulation of HIF-1α gives it an attractive role in the induction of endometrial repair factors, allowing their specific up-regulation during menstruation.

Although hypoxia is the archetypal stimulus for HIF-1α, normoxic mechanisms of HIF-1α induction have also been identified. A number of inflammatory stimuli, such as TNF-α, endotoxin, IL-1β and prostaglandins have been shown to increase HIF-1α translocation, translation or transcription to overcome proteosomal degradation in normoxic conditions at other tissue sites (Haddad and Land, 2001, Blouin et al., 2004, Fukuda et al., 2003, Frede et al., 2005). In the endometrium, HIF-1α protein has been co-localised with the EP2 receptor using dual immunohistochemistry (Critchley et al., 2006b). Furthermore, this study revealed that PGE_2 stimulated HIF-1α mRNA and protein in endometrial cells in normoxia. In contrast, other studies of endometrial tissue have failed to identify nuclear HIF-1α protein immunostaining (Zhang and Salamonsen, 2002), making its role in endometrial physiology controversial.

The working hypothesis for this chapter was that hypoxia and prostaglandins increase endometrial repair factors via induction of the transcription factor HIF-1. The aim of this chapter was to identify nuclear HIF-1α in the human endometrium by studying endometrial biopsies collected at well defined stages of the menstrual cycle. In addition, the induction of HIF-1α mRNA and protein in endometrial cells was examined. Finally, the effect of inhibition of HIF-1 on endometrial repair factor expression was investigated using HIF-1α silencing and pharmacological inhibition of HIF-1 binding.

5.2 Methods

5.2.1 Tissue collection

Endometrial tissue was collected and classified as menstrual, proliferative, early secretory, mid secretory and late secretory as described in Chapter 2.1. The endometrial biopsies used in Q-RT-PCR studies in this chapter (n=41) are detailed in table 5 (Chapter 3.2.1). Endometrial tissue samples examined for HIF-1α protein are detailed in Table 9.

Table 9. Endometrial tissue samples analysed for HIF-1α protein

Stage of Cycle	Number of biopsies	Day of Cycle	Serum oestradiol in pmol/litre Mean (range)	Serum progesterone in nmol/litre Mean (range)
Menstrual	2	2, 3	308 (102-514)	3.90 (1.24-6.49)
Proliferative	4	8-16	484 (1.3-1105)	2.07 (1.51-2.47)
Early secretory	3	16-20	684 (547-841)	52.63 (32.79-65.19)
Mid secretory	3	21-23	415 (301-613)	62.76 (42.74-82.5)
Late secretory	2	26, 29	172 (168-176)	7.87 (4.44-11.29)

5.2.2 Cell culture

Human endometrial epithelial cells (Ishikawa) were transfected with the EP2 or FP receptor (EP2S and FPS respectively) and maintained in culture as described in Chapter 2.3.3.

- Approximately 4 x 10^5 EP2S or FPS cells were seeded in 6 well plates. The following day, cells were washed in PBS and incubated in serum-free culture medium containing antibiotics and 8.4 μM indomethacin for at least 16h. To determine the contribution of HIF-1, cells were pre-treated with vehicle or 1, 2, 5 or 10nM echinomycin (an inhibitor of HIF-1 DNA binding activity) for 1 h. Cells were then stimulated with vehicle, 100nM $PGF_{2\alpha}$ or PGE_2 ± echinomycin, or placed in a hypoxic chamber (Coy laboratory products, 0.5% O_2, 5% CO_2) ± echinomycin for 8h (n=3 separate experiments).
- Approximately 1x10^6 EP2S or FPS cells were seeded in 10cm petri dishes. The next day, cells were washed in PBS and incubated with 8.4μM indomethacin as above. Cells were then treated with vehicle, 100nM PGE_2, hypoxic conditions (0.5% O_2, 5% CO_2) or with 8.4μM indomethacin and hypoxia for 2, 4, 8, and 24h. Cells were then washed with ice cold PBS, scraped into a 1ml eppendorf, centrifuged at 13000rpm for 30s and the pellet resuspended in cytoplasmic protein lysis buffer to commence the nuclear protein extraction protocol (Chapter 2.5.1).

5.2.3 HIF-1α silencing

EP2S and FPS cells were transfected with SiRNA or ShRNA constructs against HIF-1α as described in Chapter 2.9. To confirm the efficiency of HIF-1α mRNA knockdown and its specificity, cells were washed in PBS 24h after the end of the transfection period (i.e. prior to time of PG/hypoxic stimulation) and RNA extracted. To confirm knockdown at the protein level, cells were incubated in hypoxic conditions (0.5% O_2) for 8h prior to nuclear protein extraction.

5.2.4 Inhibition of NFkB

To determine the contribution of NFκB to endometrial repair factor expression, EP2S cells were seeded at a density of 1x10^5. The following day, cells were infected with

an adenovirus containing a dominant negative IκBα mutant, which maintains NFκB in a cytoplasmic location, or control adenovirus (Ad-d1703) at a total MOI of 50 for 8h. Ad-d1703 and Ad-IκBα have been described previously (Jobin et al., 1997, Henriksen et al., 2004). Cells were then serum starved with 8.4μM indomethacin for at least 16h before treatment with 100nM PGE_2 or hypoxic conditions for 6h.

5.2.5 RNA extraction and Q-RT-PCR

RNA was extracted from endometrial biopsies (n=41) and endometrial cells after culture. cDNA was prepared and Q-RT-PCR performed as detailed in general materials and methods (Chapter 2.4). Expression of mRNA for HIF-1α was measured in endometrial biopsies and IL-8, AM and VEGF mRNA levels quantified in cultured cells (see Table 3, Chapter 2 for primer/probe sequences).

5.2.6 Nuclear Protein extraction

Nuclear protein was extracted from endometrial cells and tissue biopsies, using the methodology described in Chapter 2.5.1. Nuclear protein extracts were also obtained from endometrial tissue using the protocol described in Chapter 2.5.2.

5.2.7 Immunohistochemical staining for HIF-1α

HIF-1α protein was localised in endometrium using the protocol described in Chapter 2.6. and Table 4. Endometrial explants from the proliferative phase were incubated in serum free RPMI media in a sealed hypoxic chamber (0.5% O_2, 5% CO_2, Coy laboratory products) for 24h before wax embedding. This "hypoxia exposed" endometrium was included as a positive control for HIF-1α immunohistochemistry staining.

5.2.8 Western Blot HIF-1α

HIF-1α protein was detected by Western blotting in endometrial tissue from across the menstrual cycle (n=8) using methods described in Chapter 2.5.3, 2.5.4 and 2.5.5. β-actin was detected in nuclear extracts as a housekeeping control protein.

5.2.9 Immunoprecipitation of HIF-1α

15µl of nuclear protein extract was diluted in 150µl PBS with protease inhibitors (Roche, UK). 1µl rabbit anti-human HIF-1α antibody (Santa Cruz, CA, USA) or mouse anti-human HIF-1α (NB 100-105) (Novus Biological, Cambridge, UK) was added and incubated on ice on a rocking platform for 1h. 20µg of IgG agarose beads (Santa Cruz) were added and incubated for a further 2h on ice. The samples were then centrifuged at 14000rpm for 1min, the supernatant discarded and the sample resuspended in 100µl PBS/protease inhibitor. This centrifugation process was repeated twice before resuspending the pellet in 30µl Laemmli buffer (See 2.5.3) and performing Western blotting for HIF-1α (Chapter 2.5.4 and 2.5.5).

5.2.10 Statistical analysis

For mRNA data results are expressed as fold increase, for which relative expression of mRNA in treated samples was divided by the relative expression in vehicle-treated samples. Data are presented as mean +/- SEM. Significant difference was determined using one-way ANOVA of dCt values, with Tukey's post test analysis.

5.3 Results

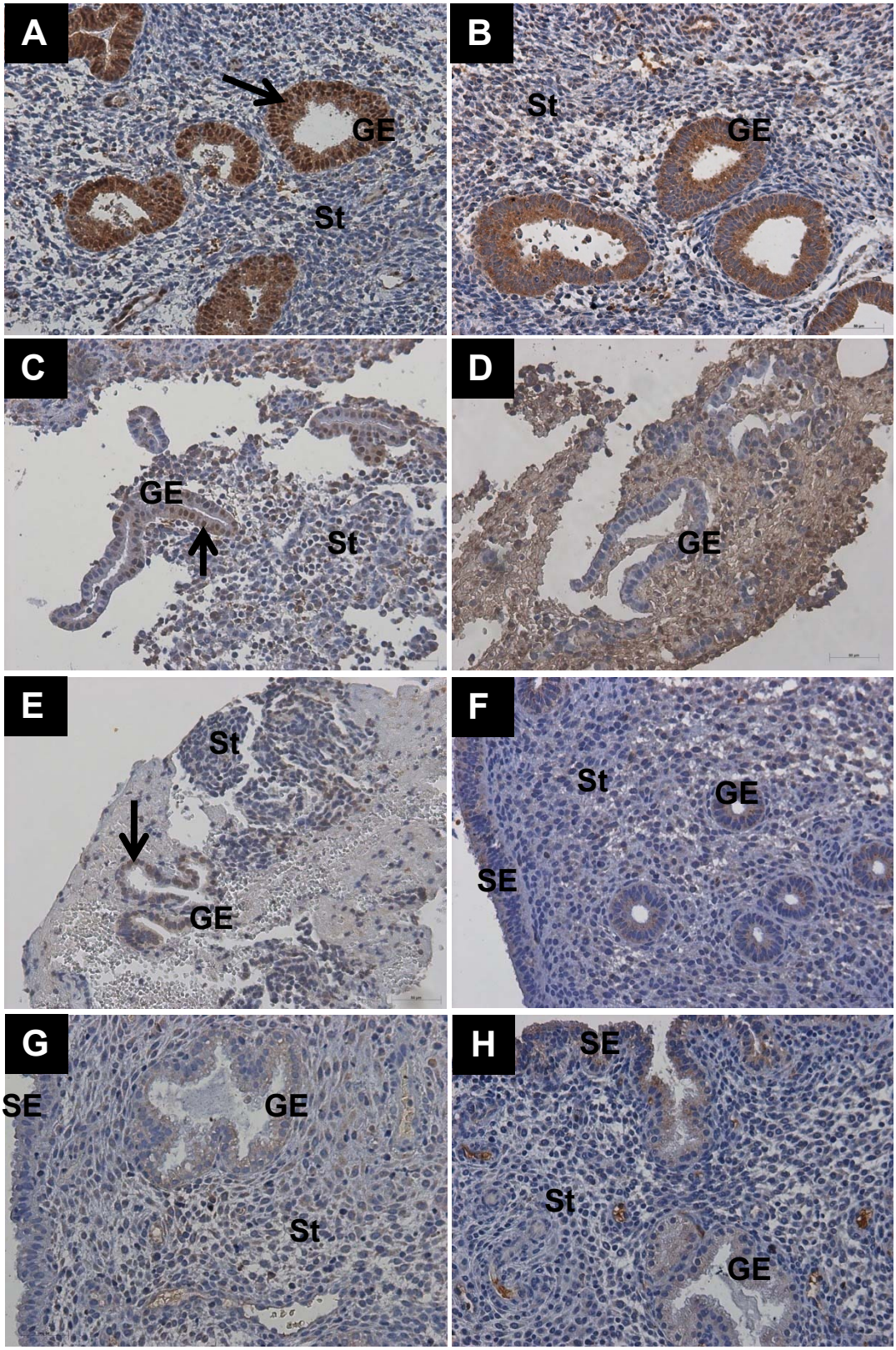
5.3.1 HIF-1α immunohistochemical staining

Endometrium from different phases of the menstrual cycle was stained with an anti-human HIF-1α rabbit polyclonal antibody. Even after optimisation, this revealed nuclear staining and non-specific cytoplasmic staining for HIF-1α (Figure 59). Proliferative endometrial tissue pre-incubated in hypoxic conditions for 24h before wax embedding was used as a positive control. HIF-1α was detected in the glandular epithelial (GE) cells and in very occasional stromal cells of this tissue (Figure 59A). When this tissue was stained with concentration matched IgG as a negative control (Figure 59B) there was strong cytoplasmic staining in the GE and some stromal cells but no nuclear staining. Staining of menstrual phase endometrium (Figure 59C and E) did show occasional nuclear staining for HIF-1α in GE cells. Assessment of the stromal cell compartment was difficult due to poor tissue quality at this cycle stage. Menstrual tissue stained with concentration matched IgG, displayed non-specific cytoplasmic staining of the stromal cells but no nuclear staining (Figure 59D). Proliferative, mid-secretory and late secretory endometrium (Figures 59E-G respectively) showed minimal cytoplasmic staining only, with no positive nuclear cells identified. Although positive nuclear staining for HIF-1α was only observed in menstrual endometrial tissue, the lack of specificity of the antibody used necessitated further analysis of endometrial HIF-1α protein (section 5.3.2).

5.3.2 HIF-1α Western blot

Due to the lack of specificity of immunohistochemical staining, a Western blot for HIF-1α was optimised to assess and quantify HIF-1α in nuclear protein extracts from endometrium at different stages of the menstrual cycle. Optimisation was carried out on nuclear extract from EP2S cells incubated in normoxic or hypoxic conditions for 24h (Figure 60A, B). Reduction of the concentration of the secondary antibody and a change of detergent wash buffer resulted in a clean band at 120kDa that was specific to hypoxic cells (Figure 60B). Unfortunately, adaptation of the cellular nuclear extraction and Western blot protocols for frozen endometrial tissue biopsies was unsuccessful (Figure 61).

Figure 59. **Immunohistochemical staining for hypoxia inducible factor-1α (HIF-1α) in the human endometrium**. **(A)** Positive control: proliferative endometrium cultured in hypoxic conditions (0.5% O_2) for 24h prior to wax embedding and stained with anti-HIF-1α antibody, **(B)** Negative control: the same hypoxic proliferative tissue stained with concentration matched IgG, **(C)** Menstrual endometrium **(D)** Negative control: the same menstrual tissue stained with IgG, **(E)** Menstrual endometrium, **(F)** Proliferative endometrium, **(G)** Mid-secretory endometrium, **(H)** Late-secretory endometrium. GE: glandular epithelial cells, SE: surface epithelial cells, St: stromal cell compartment, arrows: nuclear staining.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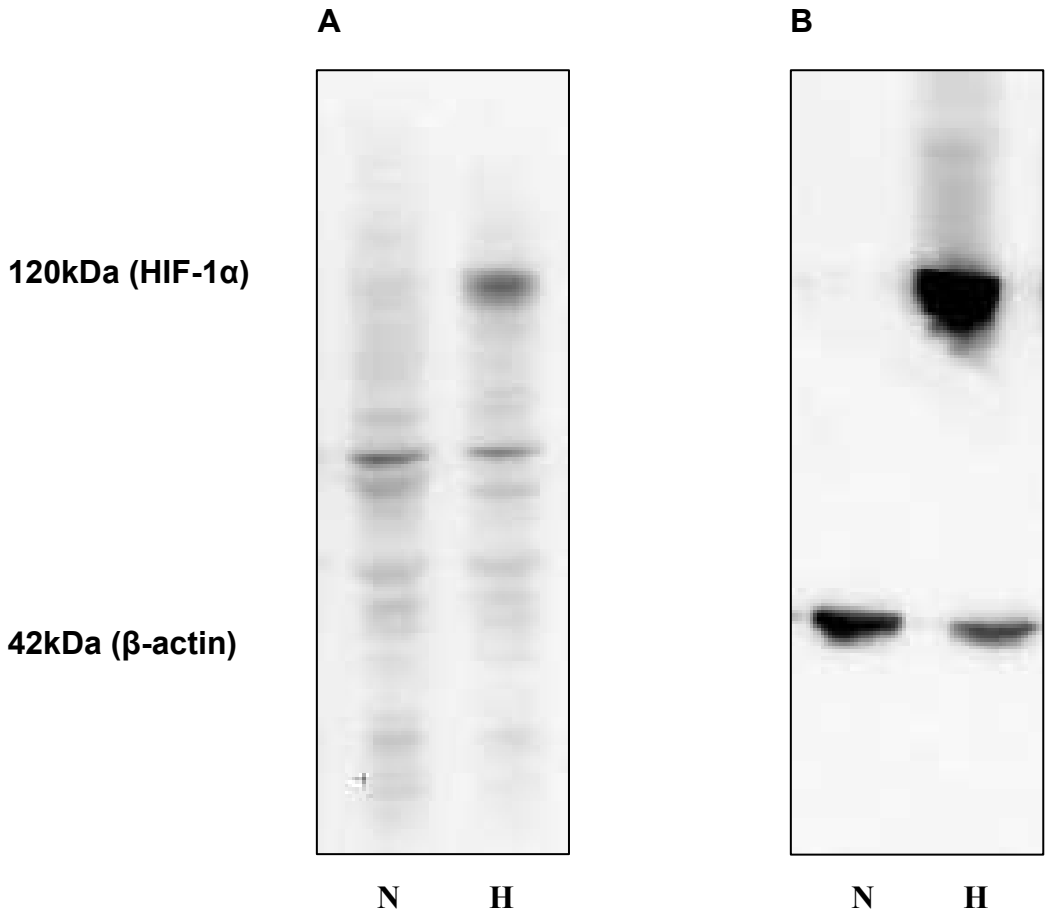


Figure 60. **Optimisation of Western blot protocol for examination of HIF-1α in endometrial cell nuclear protein extracts**. **(A)** Initial Western blot for HIF-1α in EP2S cells cultured for 24h in normoxic (N: 21% O_2) and hypoxic (H: 0.5% O_2) conditions, **(B)** Optimised Western blot for HIF-1α in EP2S cells, with beta actin as housekeeping control. Note the specific band at 120kDa when cells were incubated in hypoxic conditions, indicating stabilisation of HIF-1α.

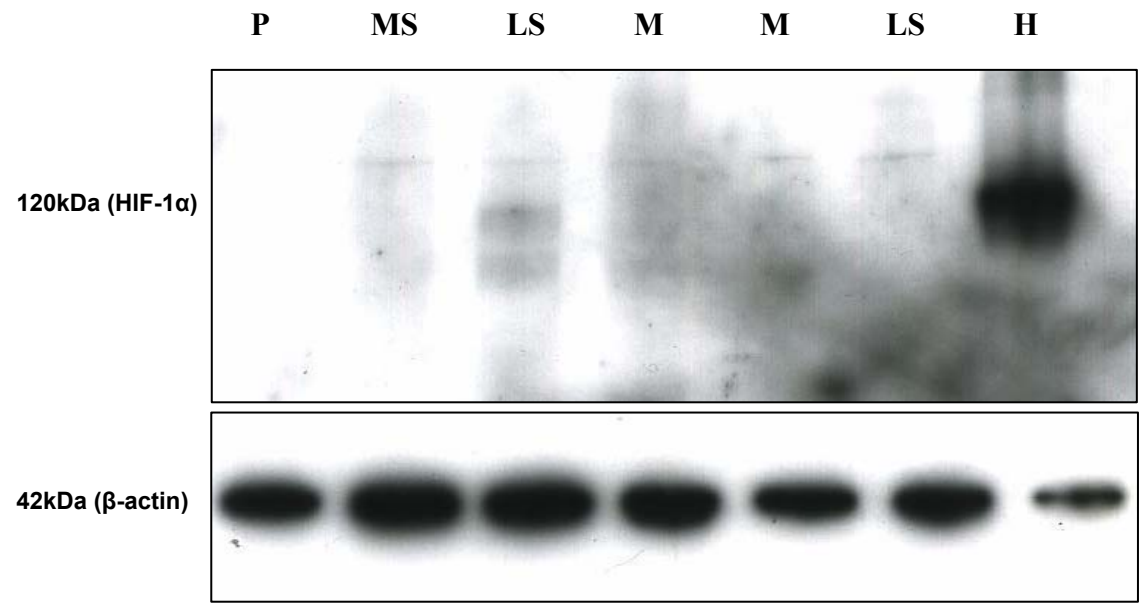


Figure 61. **Western blot for HIF-1α in human endometrial tissue**. Nuclear protein was extracted from endometrial biopsies using the protocol described in Chapter 2.5.1 before performing a Western blot for HIF-1α. Minimal HIF-1α protein was detected using these methods. M: menstrual, P: proliferative, MS: mid secretory, LS: late secretory, H: hypoxic EP2S cells as a positive control.

As endometrial HIF-1α may be present in very low levels, immunoprecipitation of HIF-1α protein from endometrial tissue nuclear protein extracts was attempted. Despite trial of two different antibody combinations and inclusion of protease inhibitors to prevent HIF-1α degradation during the IP process, this method was also unsuccessful in obtaining a clean 120kDa band and yielded inconclusive results (Figure 62).

Finally, an alternate nuclear protein extraction protocol was adapted from the Active Motif nuclear extraction kit. Using this protocol (Chapter 2.5.2), nuclear protein extract was obtained from further endometrial samples from across the menstrual cycle. When these extracts were subjected to Western blot for HIF-1α, an interesting pattern emerged. Nuclear extracts from late secretory, menstrual and very early proliferative (day 8) phase endometrium showed evidence of HIF-1α induction (Figure 63A). In contrast, there was no evidence for the presence of HIF-1α in extracts from the mid-late proliferative and early-mid secretory phases. Densitometric analysis, using β-actin as a control, revealed that maximal HIF-1α protein was present in menstrual phase endometrial extracts (Figure 63B).

5.3.3 Regulation of endometrial HIF-1α protein

Endometrial epithelial cells (EP2S) exposed to 0.5% O_2 for 2, 4, 8, and 24h demonstrated induction of HIF-1α protein (Figure 64A). In contrast, cells exposed to 100nM PGE_2 for 2, 4, 8, and 24h before nuclear protein extraction did not display a band for HIF-1α on Western blot (Figure 64B). Cells exposed to hypoxia, PGE_2 and both hypoxia and PGE_2 for 8 and 24h revealed no additional increases in HIF-1α protein with simultaneous exposure to low oxygen levels and PGE_2 (Figure 64C). To determine if the hypoxic induction of HIF-1α was dependent on prostaglandin production, EP2S cells were incubated in hypoxic conditions with and without 8.4μM indomethacin, a COX-2 inhibitor. There was no visible difference in HIF-1α protein in the presence of indomethacin for 2, 4, 8 or 24h versus hypoxia alone (Figure 65).

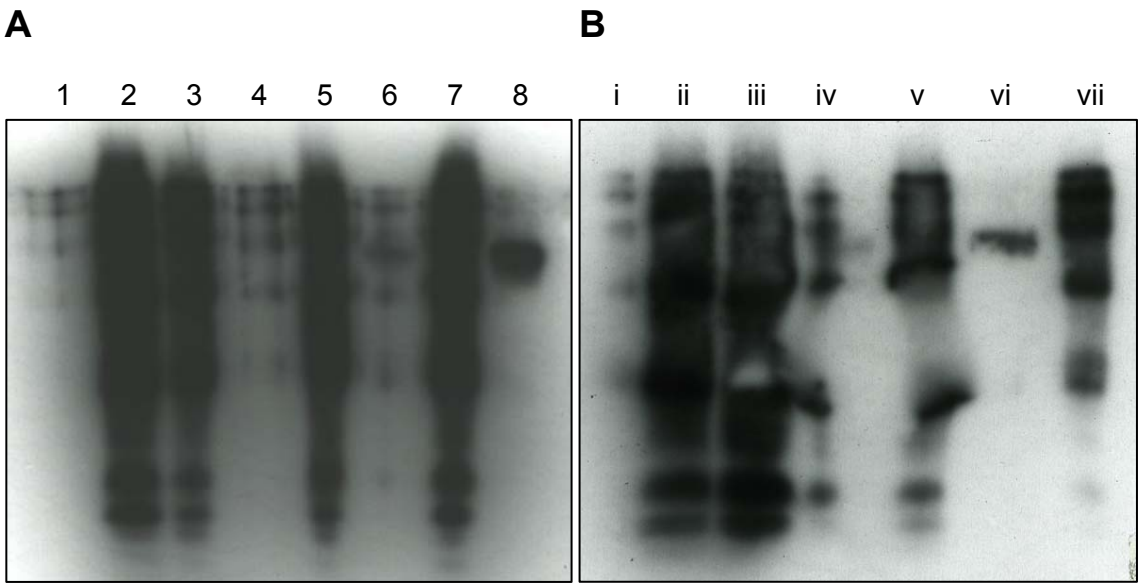


Figure 62. **Unsuccessful immunoprecipitation (IP) of HIF-1α using two different antibodies against HIF-1α (A)** Santa Cruz, CA, USA and **(B)** Novus biological, Cambridge, UK. (1) proliferative nuclear extract, (2) proliferative IP, (3) menstrual IP, (4) mid-secretory nuclear extract, (5) mid-secretory IP, (6) menstrual nuclear extract, (7) menstrual IP, (8) nuclear extract from hypoxic EP2S cells as a positive control. (i) Menstrual nuclear extract, (ii) menstrual IP, (iii) Hypoxic EP2S cells IP, (iv) menstrual nuclear extract, (v) menstrual IP, (iv) hypoxic EP2S cells nuclear extract, (vi) hypoxic EP2S cells IP.

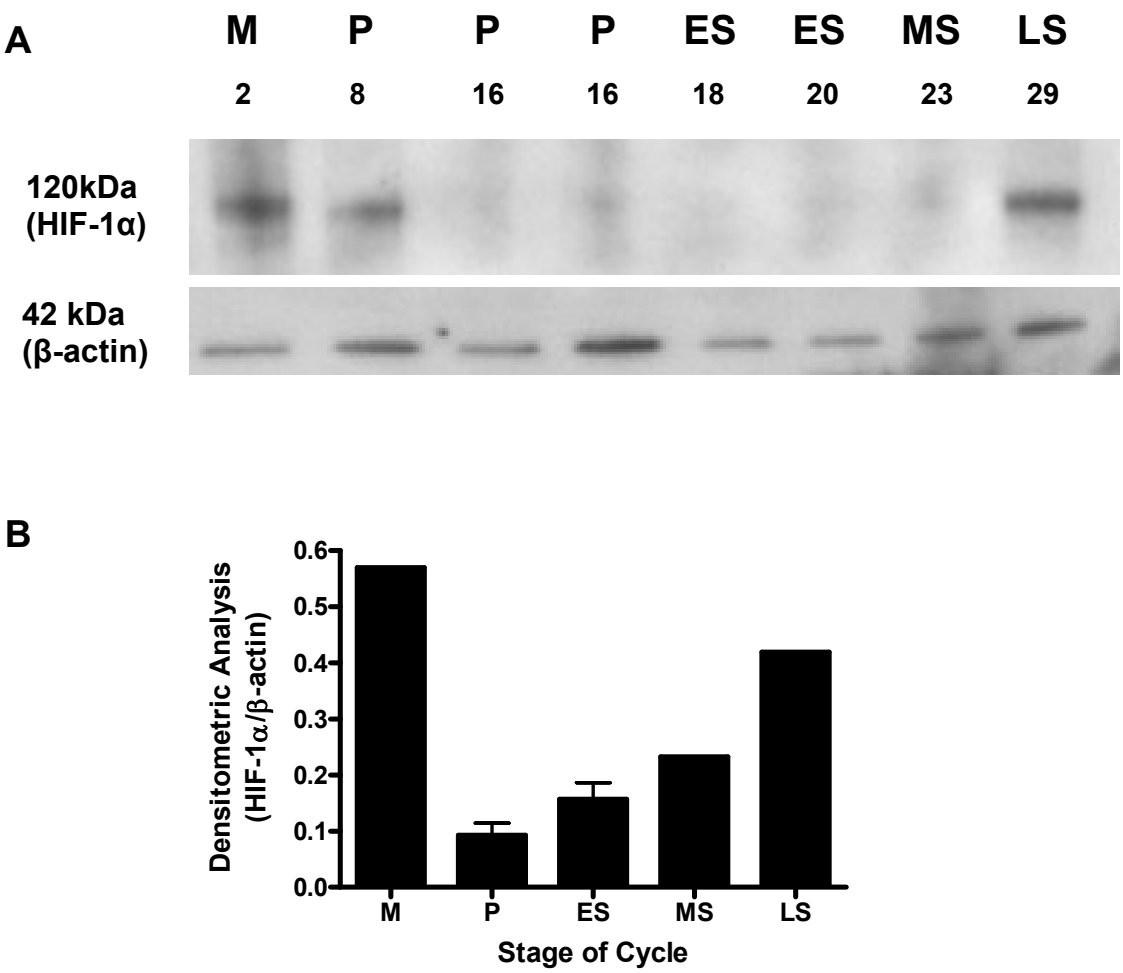
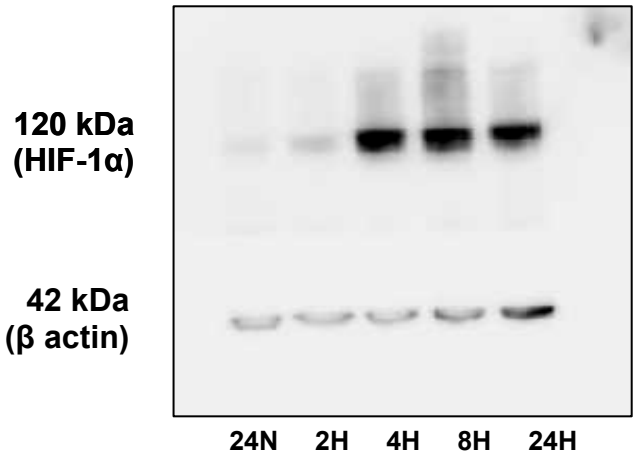


Figure 63. **The presence of HIF-1α in nuclear protein extracts from endometrial tissue collected at different times during the menstrual cycle**. (A) Western blot for HIF-1α and beta-actin in endometrial tissue from the menstrual (M), proliferative (P), early-secretory (ES), mid-secretory (MS) and late secretory (LS) phase. Numbers indicate the day of the cycle when the biopsy was collected. (B) Densitometric analysis of HIF-1α protein normalised against beta-actin.

Figure 64. **Hypoxia inducible factor -1α (HIF-1α) in human endometrial epithelial cells**. **(A)** EP2S cells were incubated in normoxic and hypoxic conditions for up to 24h before nuclear protein extraction and Western blotting for HIF-1α and β-actin. **(B)** EP2S cells were incubated with vehicle or 100nM prostaglandin E_2 (PGE_2) for up to 24h before nuclear protein extraction and Western blotting for HIF-1α and β-actin. **(C)** EP2S cells incubated with vehicle, 100nM PGE_2, hypoxic conditions and PGE_2 plus hypoxia for 8 and 24h had nuclear protein extracts subjected to Western blotting for HIF-1α and β-actin. Human endometrial stromal (HES) cells incubated in hypoxia for 24h were used as a positive control. H: hypoxia (0.5% O_2), N: normoxia (21% O_2), V: vehicle treatment.

A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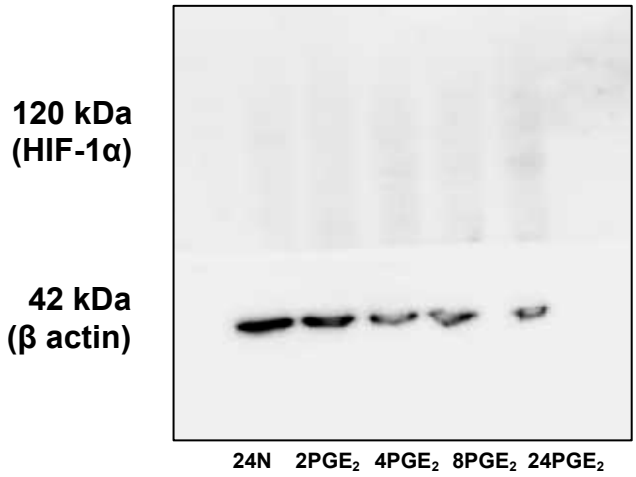
B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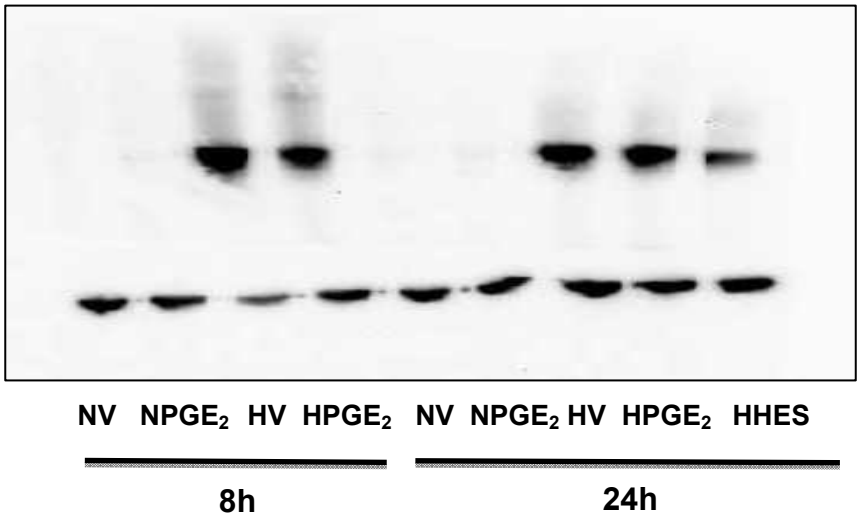
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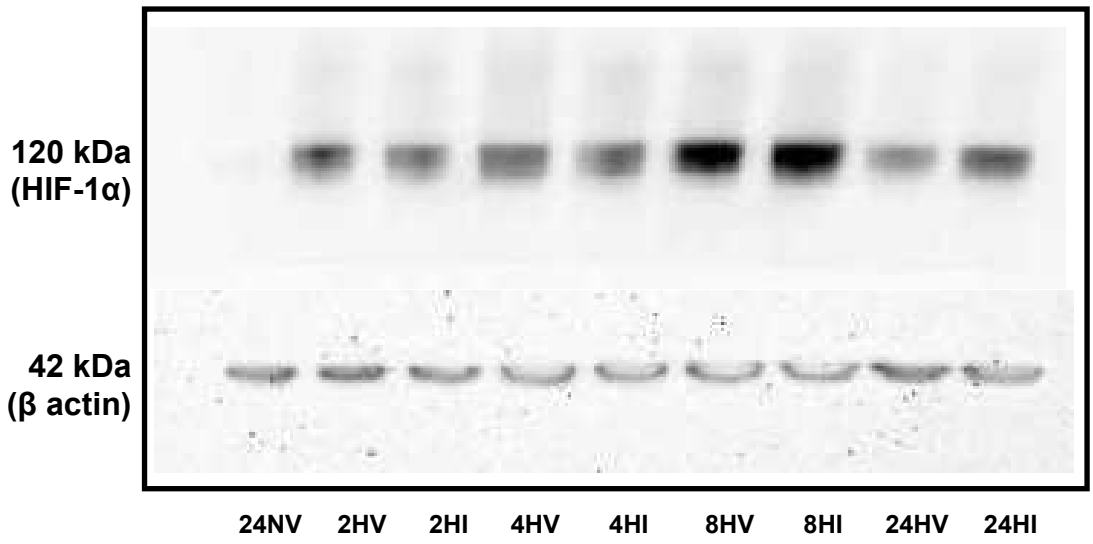


Figure 65. **Hypoxic induction of HIF-1α in EP2S cells is independent of prostaglandin production**. EP2S cells were pre-treated with vehicle or 8.4μM indomethacin (a COX-2 inhibitor) for 1h before treatments were replenished and cells placed in hypoxic conditions for up to 24h. Nuclear protein was extracted and subjected to Western blot for HIF-1α and β-actin. N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: vehicle treatment, I: 8.4μM indomethacin.

5.3.4 Regulation of endometrial HIF-1α mRNA

Endometrial epithelial cells showed no significant difference in HIF-1α mRNA when treated with vehicle, 100nM PGE_2 (EP2S cells) or $PGF_{2\alpha}$ (FPS cells), hypoxic conditions or both hypoxia and prostaglandins simultaneously (Figure 66).

5.3.5 Pharmacological inhibition of HIF-1 binding

To assess the contribution of HIF-1α to endometrial repair factor expression, endometrial epithelial cells (EP2S and FPS) were treated with echinomycin. This small molecule inhibits HIF-1 binding to hypoxic response elements on target genes, thereby preventing HIF-1-induced transcription (Kong et al., 2005).

EP2S cells co-treated with 5nM echinomycin and 100nM PGE_2 revealed a significant abrogation of IL-8 mRNA, compared to cells treated with PGE_2 alone ($p<0.05$) (Figure 67B). Echinomycin also reduced the hypoxic induction of IL-8 mRNA in these cells ($p<0.05$) (Figure 67C).

Co-treatment of FPS cells with 100nM $PGF_{2\alpha}$ and 1-10nM echinomycin had no significant effect on AM mRNA (Figure 68A). In contrast, echinomycin prevented AM up-regulation by hypoxia in a dose-dependent manner (Figure 68B). AM expression was significantly reduced in cells treated with hypoxia and 5nM-10nM echinomycin when compared to hypoxia treatment in the absence of echinomycin ($p<0.01$ and $p<0.001$ respectively).

FPS cells in normoxia revealed a significant reduction of VEGF mRNA when co-treated with $PGF_{2\alpha}$ and 5-10nM echinomycin, versus $PGF_{2\alpha}$ alone ($p<0.01$) (Figure 69A). Hypoxic induction of VEGF mRNA was significantly abrogated by 5nM and 10nM echinomycin ($p<0.01$, $p<0.001$ respectively) (Figure 69B).

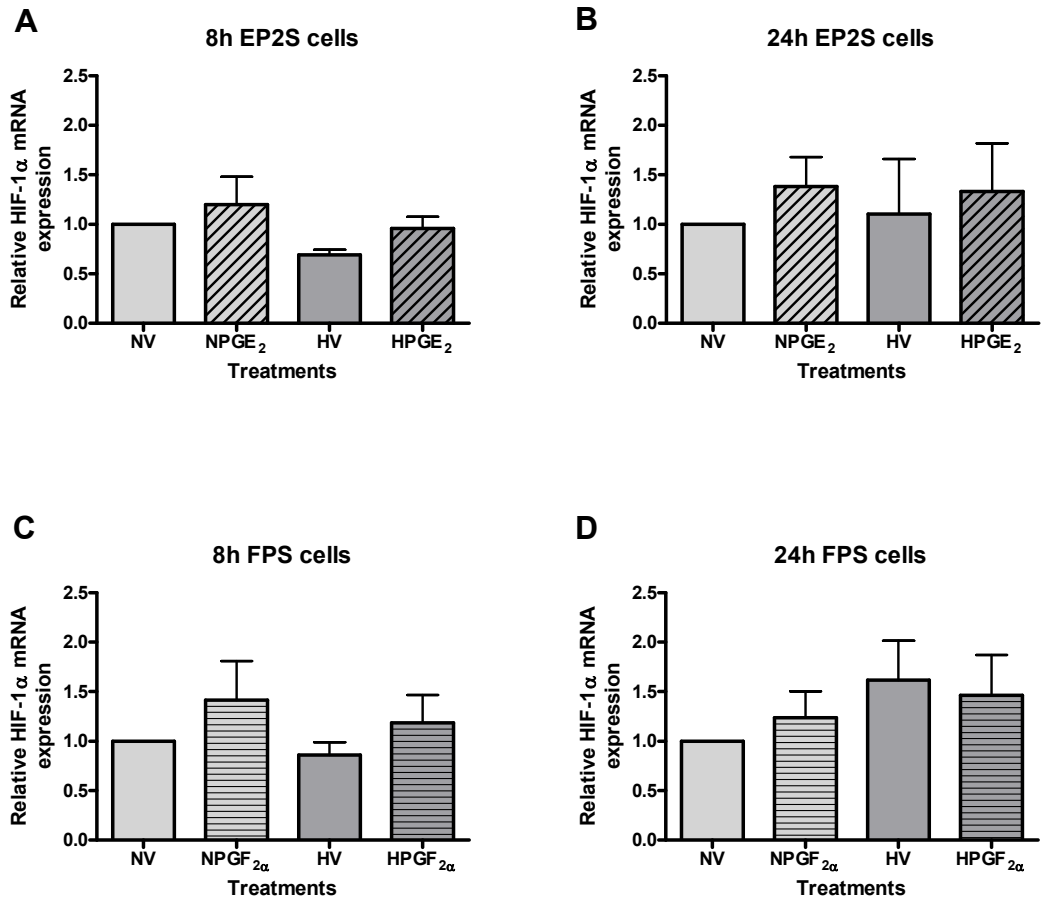


Figure 66. **The regulation of HIF-1α mRNA by hypoxia and prostaglandins.** **(A)** HIF-1α mRNA in EP2S cells treated with vehicle, 100nM prostaglandin E_2 (PGE_2), hypoxic conditions or PGE_2 and hypoxia for 8h. **(B)** HIF-1α mRNA in EP2S cells treated with vehicle, 100nM prostaglandin E_2 (PGE_2), hypoxic conditions or PGE_2 and hypoxia for 24h. **(C)** HIF-1α mRNA expression in FPS cells treated with vehicle, 100nM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), hypoxia or both $PGF_{2\alpha}$ and hypoxia for 8h. **(D)** HIF-1α mRNA in FPS cells treated with vehicle, 100nM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), hypoxia or both $PGF_{2\alpha}$ and hypoxia for 24h. V: vehicle, H: hypoxia (0.5% O_2), N: normoxia (21% O_2).

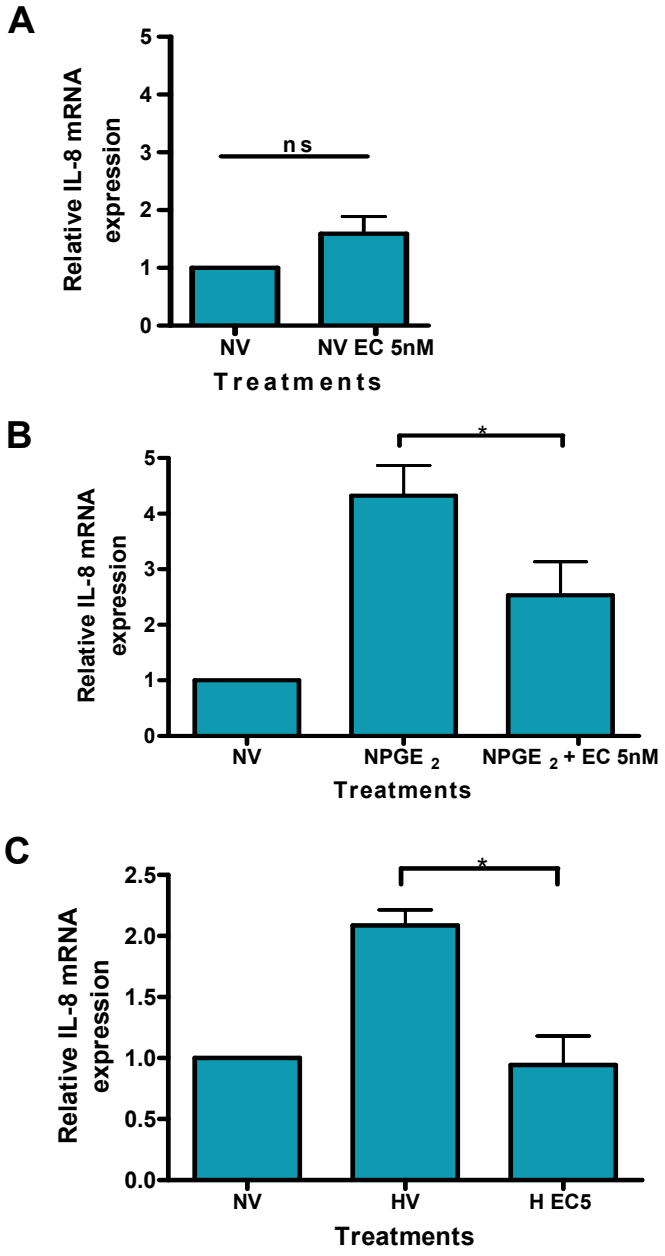


Figure 67. **The impact of pharmacological inhibition of HIF-1α on endometrial cell IL-8 mRNA**. **(A)** EP2S cells incubated with and without 5nM echinomycin in normoxic conditions showed no significant difference in IL-8 mRNA expression. **(B)** Echinomycin significantly abrogated prostaglandin E_2 (PGE_2) induced IL-8 mRNA. **(C)** Hypoxia induced IL-8 mRNA was significantly reduced when cells were treated with echinomycin. N: normoxia (21% O_2), H: hypoxia (0.5% O_2), V: vehicle, EC: 5nM echinomycin. ns: non-significant, *$p<0.05$.

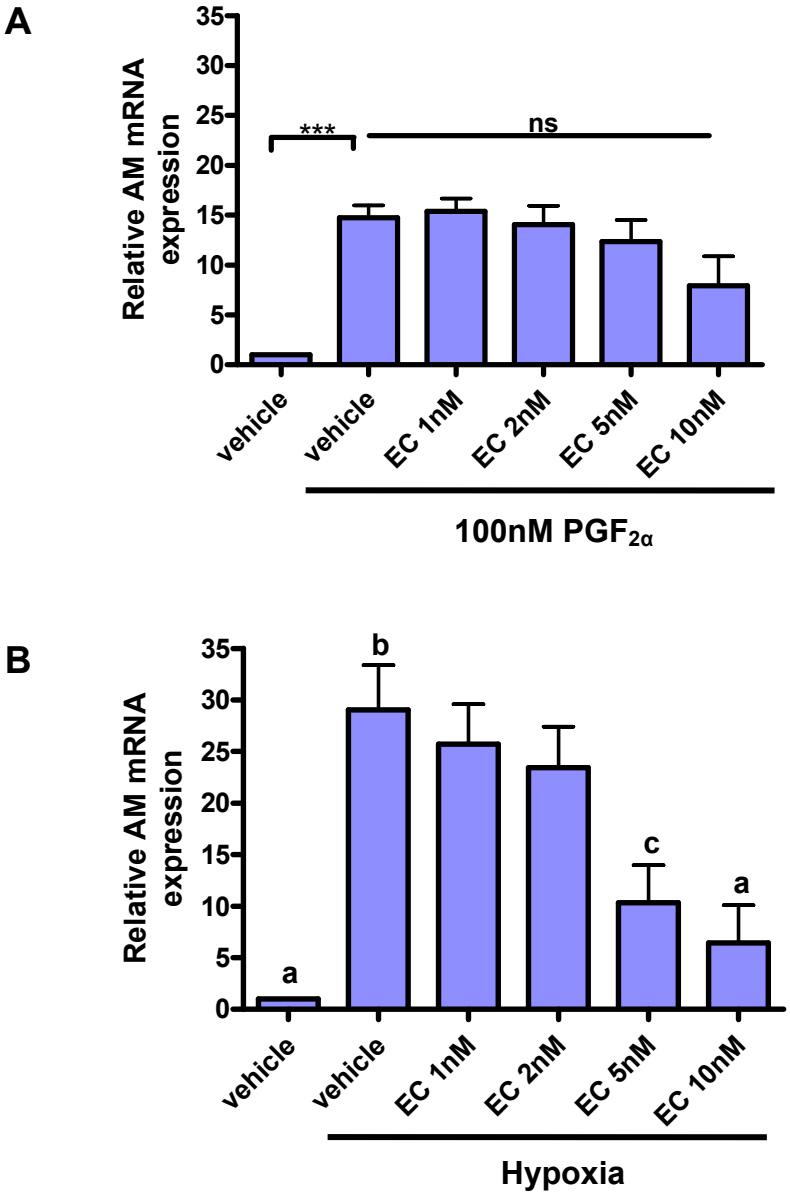


Figure 68. **The impact of pharmacological inhibition of HIF-1α on adrenomedullin (AM) mRNA in endometrial epithelial cells (FPS cells).** (A) Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induced AM mRNA was not significantly reduced when cells were co-treated with 1-10nM echinomycin. (B) Hypoxia induced AM mRNA expression was significantly abrogated by co-treatment of cells with 5 and 10nM echinomycin. EC: echinomycin, ns: non-significant, ***$p<0.001$, a-b $p<0.001$, b-c $p<0.01$.

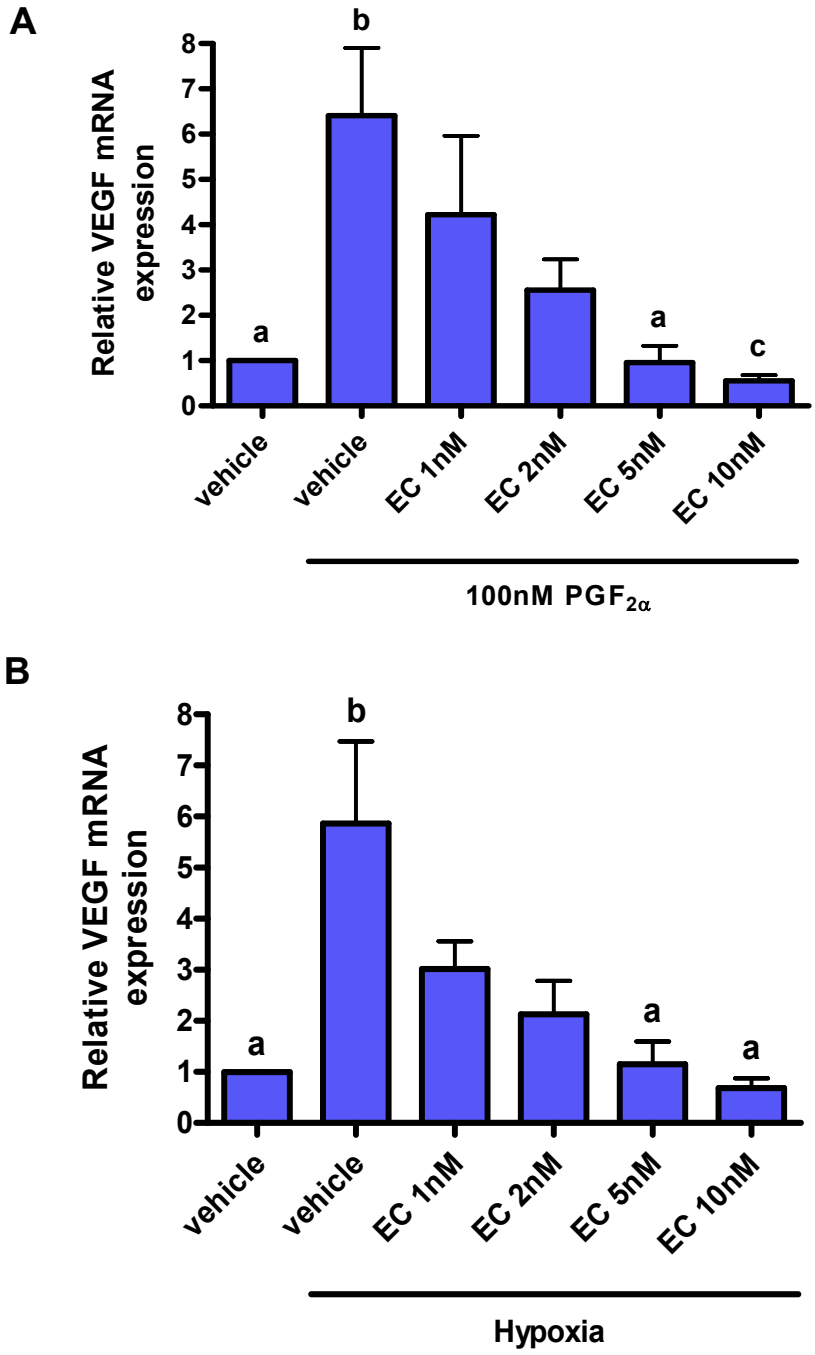


Figure 69. **The impact of pharmacological inhibition of HIF-1α on vascular endothelial growth factor (VEGF) mRNA in FPS cells.** (A) Co-treatment of FPS cells with 100nM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 5-10nM echinomycin (EC) significantly abrogated the $PGF_{2\alpha}$ mediated induction of VEGF expression. (B) Hypoxia induced VEGF mRNA was also significantly reduced with treatment of cells with echinomycin. a-b $p<0.01$, a-c $p<0.001$.

5.3.6 Silencing of HIF-1α with RNA interference

To confirm the findings obtained after pharmacological inhibition of HIF-1 binding, HIF-1α was silenced using RNA interference. First, the transfection efficiency of siRNA constructs into EP2S cells was tested using siGLO, an oligonucleotide labelled with 6-FAM fluorescent indicator. Even with siGLO concentrations of 100nM, there were few fluorescently labelled cells 36h after transfection (Figure 70). Increased concentration of HiPerfect$^{(R)}$ transfection reagent and lengthened transfection times had no effect on the amount of cells transfected with siGLO.

As SiRNA transfection efficiency was found to be suboptimal, short-hairpin RNA sequences were transfected into EP2S and FPS cells using a plasmid vector in a lentivirus. A lentivirus containing green fluorescent protein (GFP) was incubated with EP2S cells at a MOI of 1, 2, 5, and 10. This titre experiment revealed maximal transfection efficiency at an MOI of 10 (Figure 71).

Based on these findings, two different ShRNA sequences against HIF-1α (HIF-1α/Sh1470 and HIF-1α/Sh2192) were transfected into EP2S and FPS cells at an MOI of 10. Figure 72 shows the transfection efficiency of EP2S cells transfected with no construct (A-C), HIF-1α/Sh1470 (D-F), HIF-1α/Sh2192 (G-I) and HIF-1α/ShSCR (J-L). FPS cells had a very similar pattern of transfection (data not shown).
HIF-1α silencing in EP2S cells was confirmed by Western blot analysis (Figure 73A), revealing a visible reduction in HIF-1α protein with both HIF-1α/ShRNA constructs compared to untransfected cells and cells transfected with HIF-1α/ShSCR. These findings were confirmed at the mRNA level using Taqman Q-RT-PCR (Figure 73B). Specificity of the EP2S cell HIF-1α knockdown was confirmed with measurement of Lamin A/C mRNA levels, which showed no significant differences with transfection of any construct (Figure 73C). Very similar findings were found on transfection of FPS cells (Figure 74A-C).

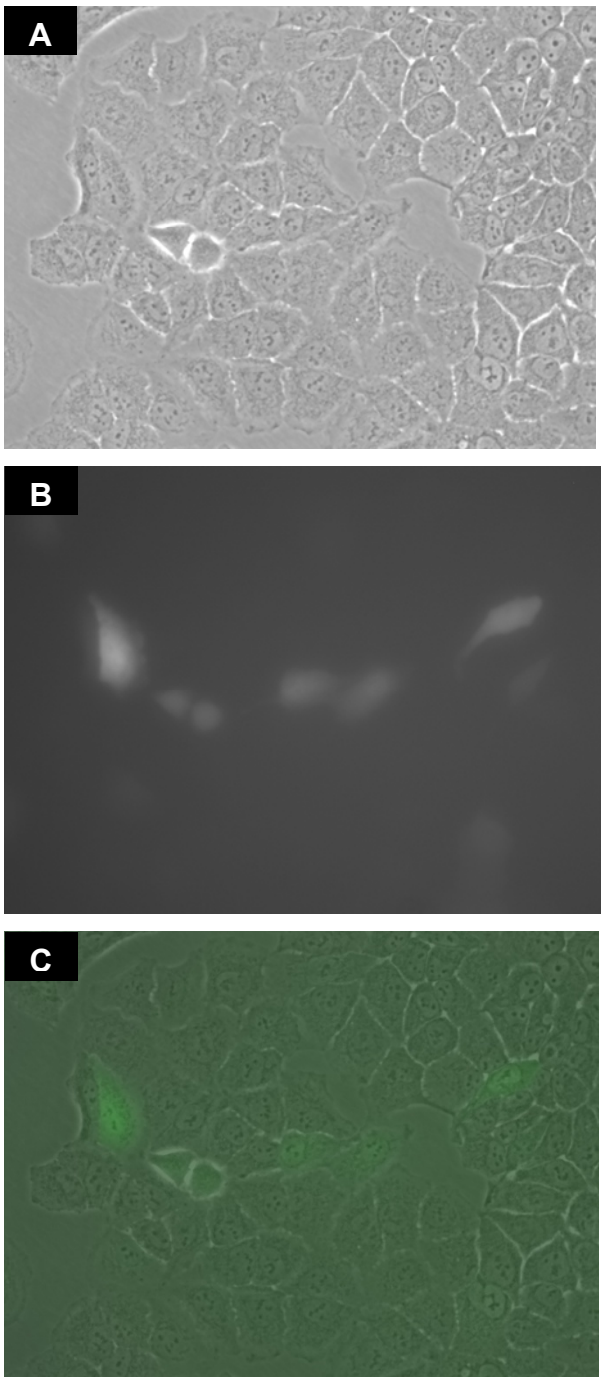


Figure 70. **Transfection of EP2S cells with short interfering RNA (siRNA) constructs**. EP2S cells were incubated with 100nM SiGLO and 1:100 HiPerfect transfection reagent for 8h and photographed 36h after transfection. **(A)** Phase contrast image **(B)** GFP expression **(C)** Superimposed images to show number of cells expressing GFP.

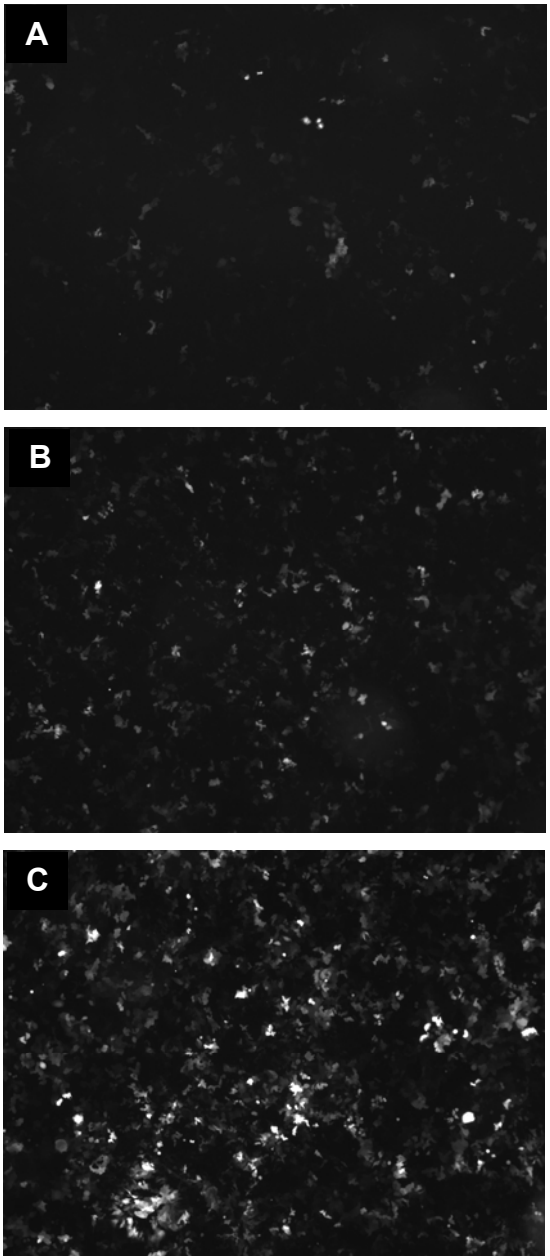


Figure 71. **Transfection of EP2S cells with short hairpin RNA (ShRNA) constructs.** ShRNA constructs labelled with green fluorescent protein (GFP) were incorporated into lentiviral vectors and incubated with EP2S cells for 24h at a multiplicity of infection of **(A)** 2, **(B)** 5 or **(C)** 10. Photographs were taken 24h after transfection using Axiovert 200 microscope (Zeiss, Welwyn Garden City, U.K.).

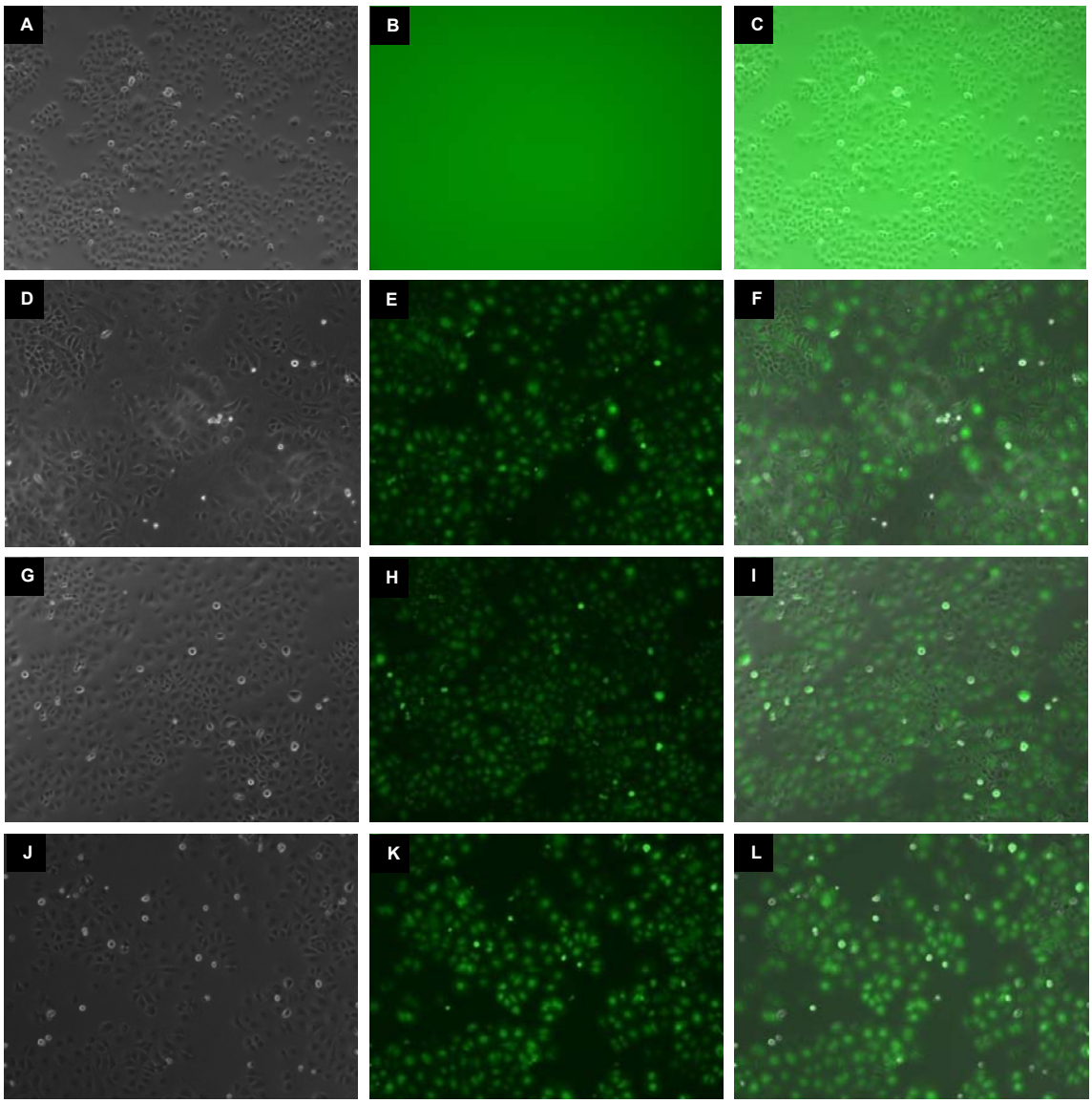
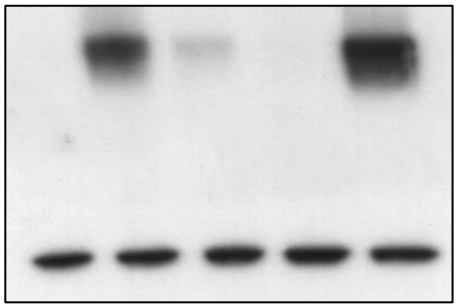


Figure 72. **Confirmation of transfection efficiency of ShRNA constructs against HIF-1α in EP2S cells**. **(A)** Untransfected control cells in phase contrast **(B)** GFP expression in untransfected control cells **(C)** Superimposed images of untransfected control cells **(D)** Cells transfected with HIF-1α/Sh1470 in phase contrast **(E)** GFP expression in cells transfected with HIF-1α/Sh1470 **(F)** Superimposed images of cells transfected with HIF-1α/Sh1470 **(G)** Cells transfected with HIF-1α/Sh2192 in phase contrast **(H)** GFP expression in cells transfected with HIF-1α/Sh2192 **(I)** Superimposed images of cells transfected with HIF-1α/Sh2192 **(J)** Cells transfected with ShSCR in phase contrast **(K)** GFP expression in cells transfected with ShSCR **(L)** Superimposed images of cells transfected with ShSCR.

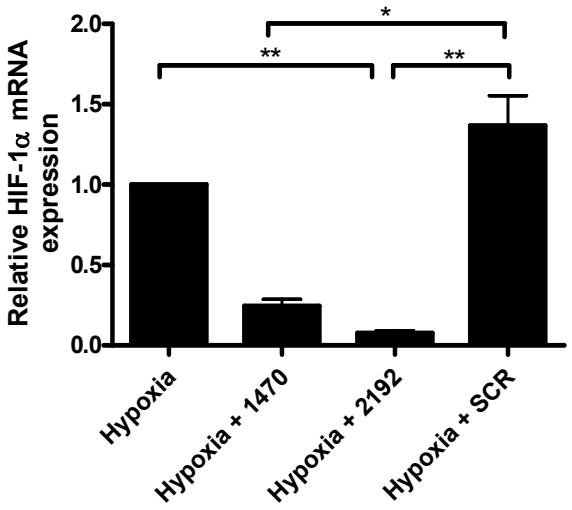
Figure 73. **Confirmation of hypoxia inducible factor-1α (HIF-1α) silencing in endometrial epithelial (EP2S) cells. (A)** Confirmation of HIF-1α protein knockdown by two short hairpin sequences against HIF-1α (HIF-1/Sh1470 and HIF-1α/Sh2192), compared to a scrambled sequence (ShSCR) or untransfected cells after 8h in hypoxic conditions (0.5% O_2). **(B)** Confirmation of HIF-1α mRNA knockdown by the same sequences. (C) Specificity of the knockdown was confirmed by examining Lamin A/C mRNA, which showed no significant changes with transfection of any construct. *$p<0.05$ **$p<0.01$, ns non-significant.

A



Hypoxia	-	+	+	+	+
HIF-1α ShRNA 1470	-	-	+	-	-
HIF-1α ShRNA 2192	-	-	-	+	-
SCR ShRNA	-	-	-	-	+

B



C

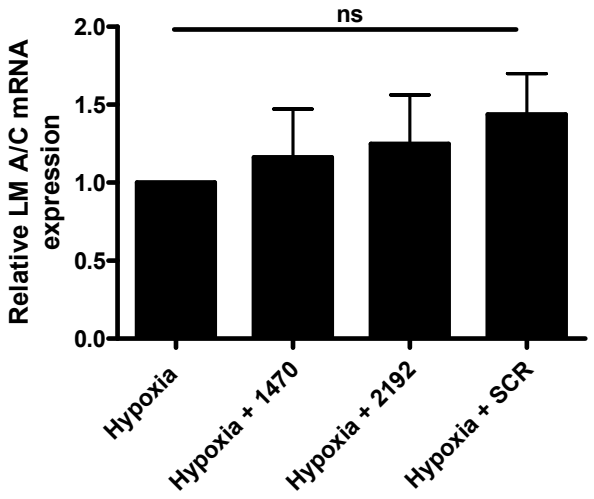
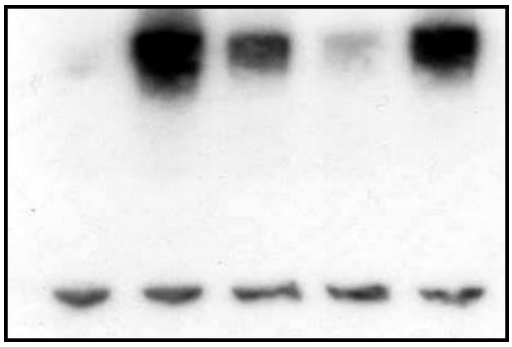


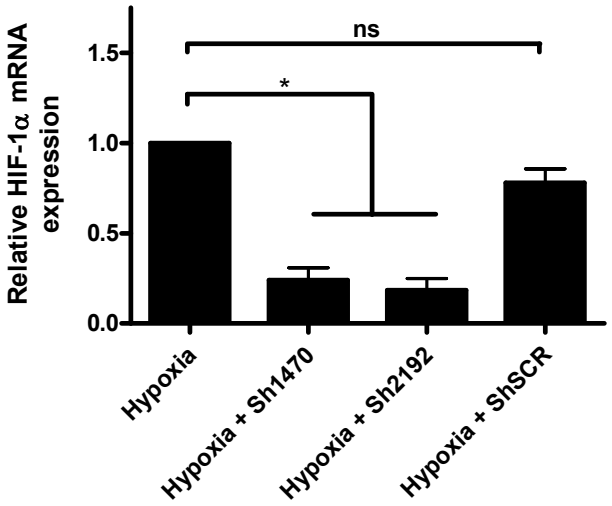
Figure 74. **Confirmation of hypoxia inducible factor silencing in FPS cells.** **(A)** Confirmation of HIF-1α protein knockdown by two short hairpin sequences against HIF-1α (HIF-1/Sh1470 and HIF-1α/Sh2192), compared to a scrambled sequence (ShSCR) or untransfected cells after 8h in hypoxic conditions (0.5% O_2). **(B)** Confirmation of HIF-1α mRNA knockdown by the same sequences. **(C)** Specificity of the knockdown was confirmed by examining Lamin A/C mRNA expression, which showed no significant changes with transfection of any construct. *$p<0.05$, **ns** non-significant.

A

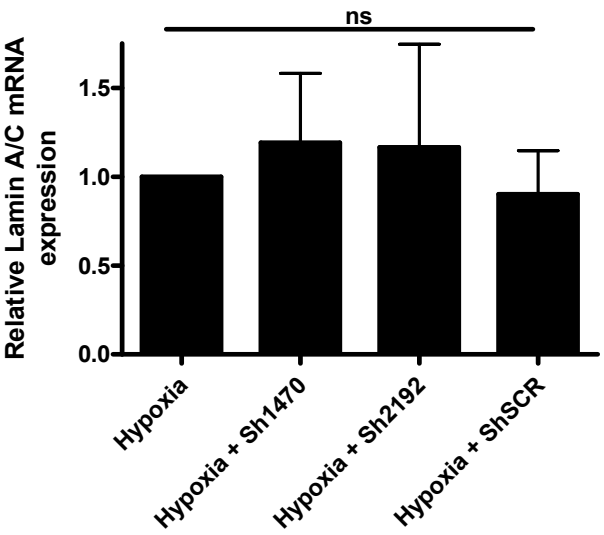


Hypoxia	-	+	+	+	+
HIF-1α ShRNA 1470	-	-	+	-	-
HIF-1α ShRNA 2192	-	-	-	+	-
SCR ShRNA	-	-	-	-	+

B



C



IL-8 mRNA did not differ significantly between untransfected EP2S cells and cells transfected with a scrambled ShRNA sequence when exposed to PGE_2 or hypoxia (Figure 75). Silencing of HIF-1α did not significantly alter PGE_2 mediated IL-8 mRNA when compared to untransfected cells. There was a reduction in IL-8 mRNA with HIF-1α/Sh1470 transfection but this was only significantly reduced compared to ShSCR transfected cells ($p<0.001$). Hypoxia mediated IL-8 mRNA was significantly reduced upon transfection with HIF-1α/Sh1470 versus non-transfected cells ($p<0.05$) and those transfected with ShSCR ($p<0.01$). Transfection of the second ShRNA sequence against HIF-1α (HIF-1α/Sh2192) did not result in a significant change in hypoxia mediated IL-8 mRNA expression when compared to untransfected cells or ShSCR transfected cells (Figure 75).

Consistent with data generated using echinomycin, HIF-1α silencing had no significant impact on $PGF_{2\alpha}$ induced AM mRNA in FPS cells (Figure 76A). However, hypoxic induction of AM was significantly lower in those cells in which HIF-1α was silenced, when compared to untransfected cells or cells transfected with HIF-1α/ShSCR ($p<0.05$) (Figure 76B).

In discordance with the data generated using echinomycin, HIF-1α silencing in FPS cells resulted in no significant differences in $PGF_{2\alpha}$-induced VEGF mRNA expression (Figure 77A). Hypoxic induction of VEGF mRNA was significantly abrogated in cells transfected with HIF-1α/Sh1470 and HIF-1α/Sh2192 when compared to untransfected or HIF-1α/ShSCR transfected FPS cells (Figure 77B).

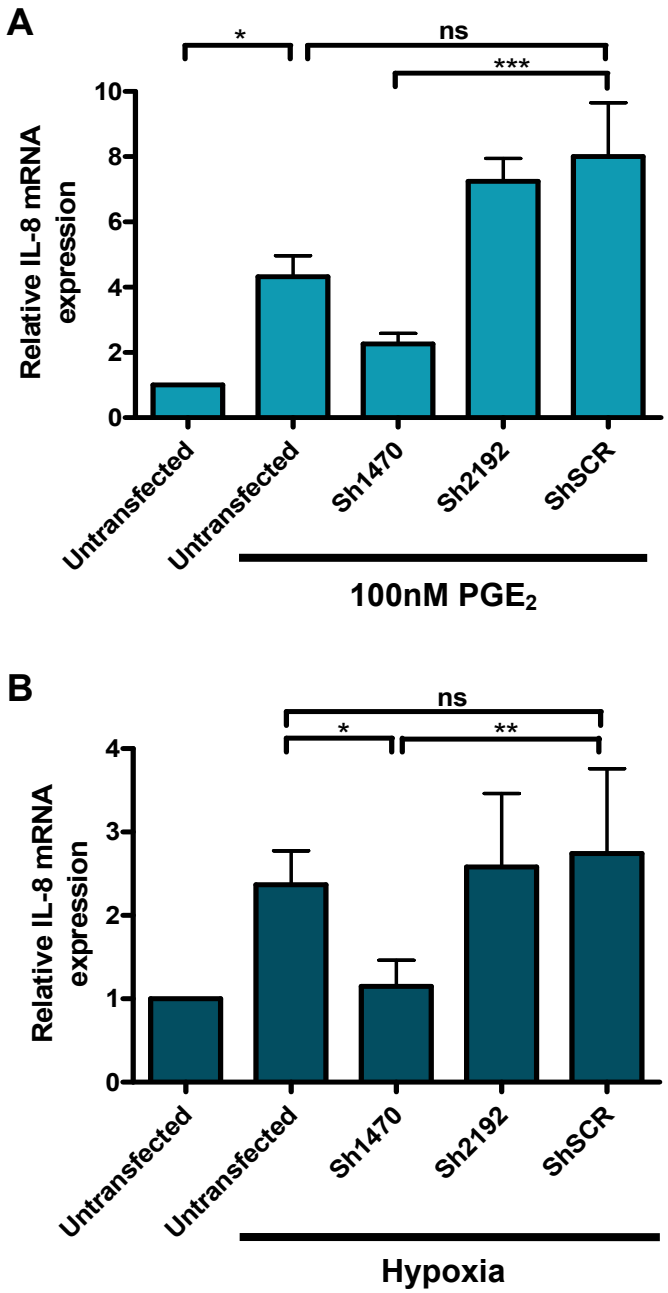


Figure 75. **The impact of hypoxia inducible factor-1α (HIF-1α) silencing on EP2S cell IL-8 mRNA. (A)** Silencing of HIF-1α with two short hairpin sequences against HIF-1α (Sh1470 and Sh2192) had no significant effect on prostaglandin E_2 (PGE_2) mediated IL-8 mRNA when compared to untransfected cells. **(B)** Hypoxia mediated IL-8 mRNA expression was significantly reduced by transfection with Sh1470 but not Sh2192. ShSCR: scrambled ShRNA sequence, hypoxia: 0.5% O_2, ns: non-significant, *p<0.05, **p<0.01, ***p<0.001.

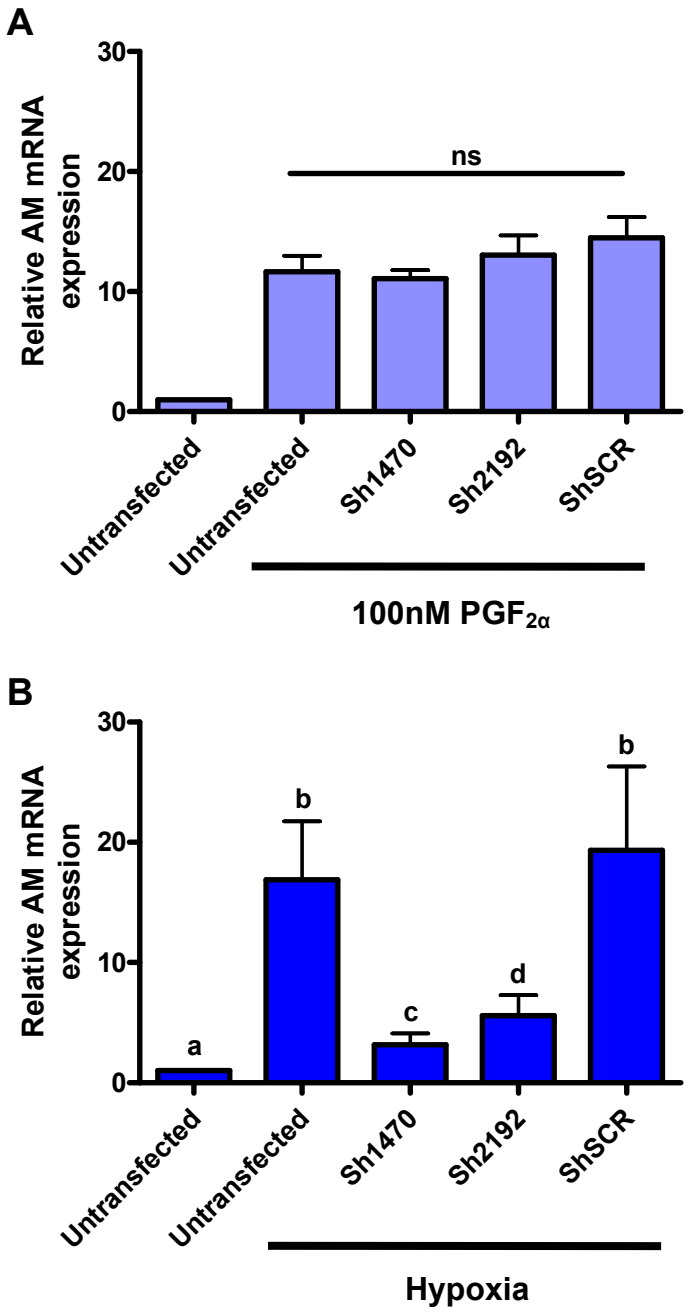


Figure 76. **The impact of hypoxia inducible factor-1α (HIF-1α) silencing on FPS cell adrenomedullin (AM) mRNA. (A)** Silencing of HIF-1α with two different ShRNA constructs (Sh1470 and Sh2192) had no significant impact on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induced AM mRNA. **(B)** Hypoxia induced AM mRNA was significantly reduced by HIF-1α silencing when compared to untransfected cells or cells transfected with a scrambled sequence (ShSCR). **ns**: non-significant, **a-b** $p<0.001$, **b-c** $p<0.01$, **b-d** $p<0.05$.

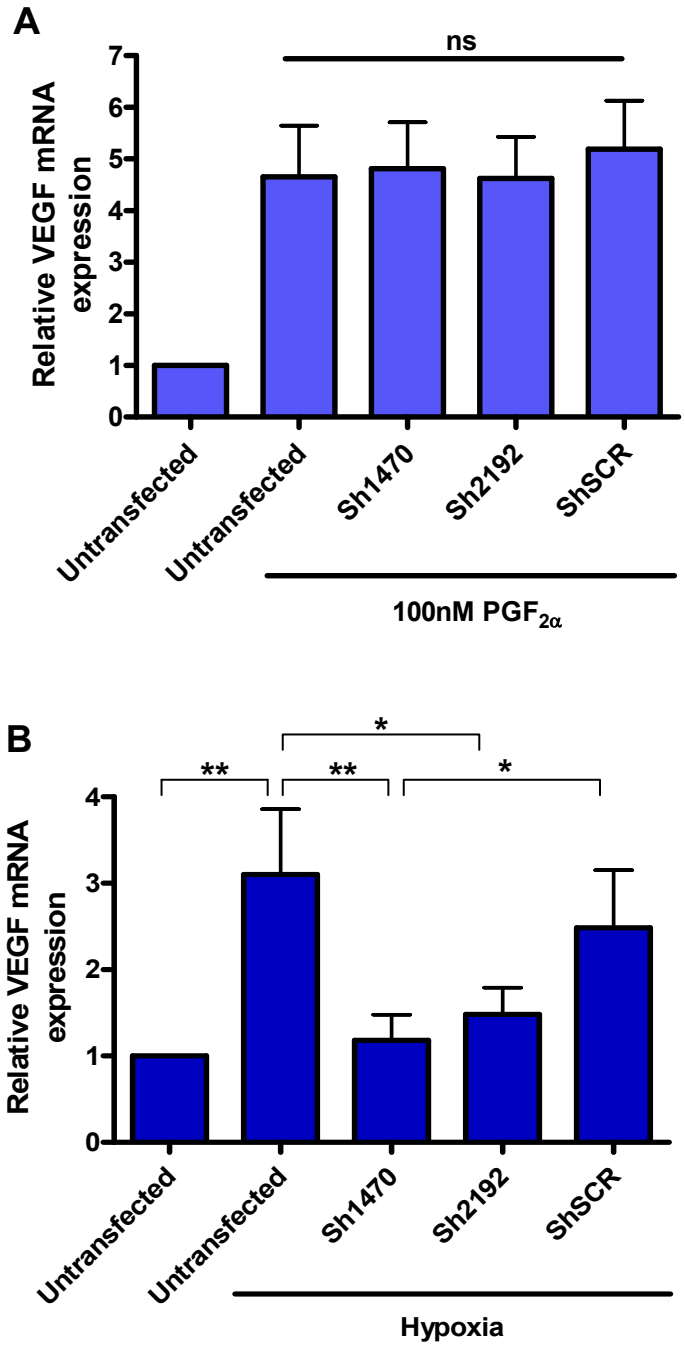
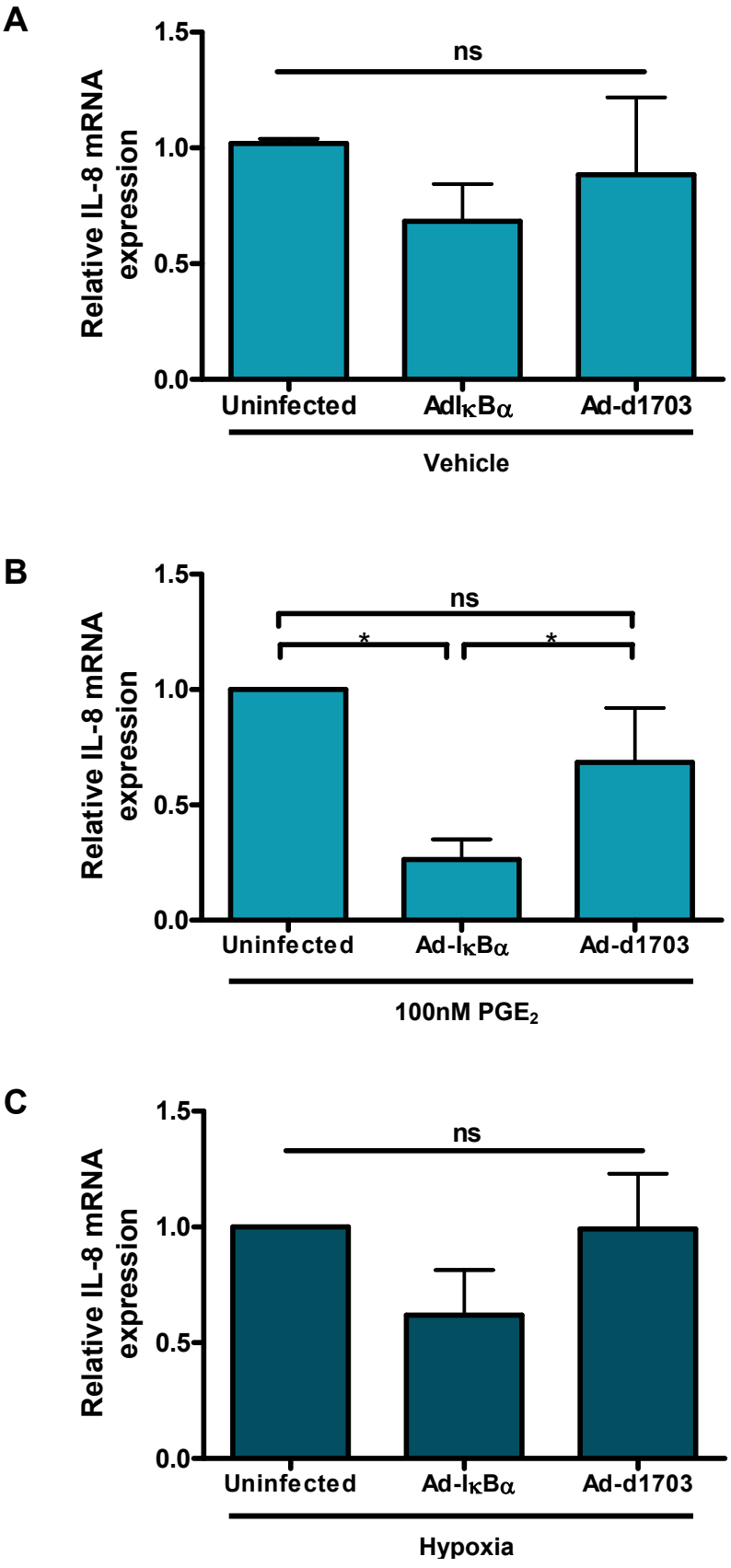


Figure 77. **The impact of hypoxia inducible factor-1α (HIF-1α) silencing on FPS cell vascular endothelial growth factor (VEGF) mRNA expression**. (A) Silencing of HIF-1α with two different ShRNA constructs (Sh1470 and Sh2192) had no significant impact on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induced VEGF mRNA expression. (B) Hypoxia induced VEGF mRNA was significantly reduced by HIF-1α silencing when compared to untransfected cells or cells transfected with a scrambled sequence (ShSCR). ns: non-significant, *$p<0.05$, **$p<0.01$.

5.3.7 Inhibition of NFkB in EP2S cells

To determine the contribution of NFκB to the up-regulation of endometrial IL-8, cells were infected with a dominant negative inhibitor of NFκB, adenovirus IκBα (Ad-IκBα) and subsequently cultured with vehicle, PGE_2 or in hypoxic conditions for 6h. Infection of cells with Ad-IκBα resulted in significant reduction of PGE_2 induced IL-8 mRNA ($p<0.05$), when compared to uninfected cells or cells infected with control adenovirus-d1730 (Figure 78B). Hypoxia induced IL-8 mRNA was not significantly affected by inhibition of NFκB (Figure 78C).

Figure 78. **The impact of inhibition of nuclear factor kappa B (NF-κB) on IL-8 mRNA in endometrial epithelial cells (EP2S). (A)** Infection of EP2S cells with a dominant-negative inhibitor of NF-κB (Ad-IκBα) or control adenovirus (Ad-d1730) had no significant impact on basal IL-8 mRNA. **(B)** Cells infected with Ad-IκBα demonstrated significant abrogation of prostaglandin E_2 (PGE_2) induced IL-8 mRNA when compared to uninfected cells or those infected with Ad-d1703. **(C)** Infection of cells with Ad- IκBα had no significant effect on the hypoxic induction of IL-8 expression. All experiments n=3, ns: non-significant, *$p<0.05$.


A
Relative IL-8 mRNA expression
1.5
1.0
0.5
0.0
ns
Uninfected
AdIκBα
Ad-d1703
Vehicle
B
Relative IL-8 mRNA expression
1.5
1.0
0.5
0.0
ns
*
*
Uninfected
Ad-IκBα
Ad-d1703
100nM PGE2
C
Relative IL-8 mRNA expression
1.5
1.0
0.5
0.0
ns
Uninfected
Ad-IκBα
Ad-d1703
Hypoxia

5.4 Discussion

These data confirm the presence of nuclear HIF-1α protein in the human endometrium. Importantly, HIF-1α could only be detected during the perimenstrual phase. In addition, novel results detailed in this chapter demonstrate two pathways of endometrial repair factor regulation; a hypoxia mediated pathway that is dependent upon HIF-1α and a HIF-1α independent pathway that is activated by $PGF_{2\alpha}$.

5.4.1 Endometrial HIF

HIF-1α has previously been detected in the human endometrium. In a study of angiogenesis in endometrial carcinoma tissue, normal endometrial controls demonstrated positive nuclear HIF-1α immunostaining (Sivridis et al., 2002). HIF-1α was detected in the glandular epithelial and stromal cells of very early proliferative samples, with decreased staining in tissues from the mid-late proliferative and secretory phases. In a study of normal endometrial tissue from across the menstrual cycle, HIF-1α was detected by immunohistochemistry exclusively in the secretory and menstrual phases, with maximal levels after progesterone withdrawal (Critchley et al., 2006b). A further study of normal endometrium from the menstrual and proliferative phases revealed maximal positive immunohistochemical HIF-1α staining in the glandular and stromal compartments of endometrium from cycle days 2-5 (Punyadeera et al., 2006). However, another study found very little staining for HIF-1α in endometrial tissue, regardless of cycle stage (Zhang and Salamonsen, 2002). These authors reported that 20 out of 34 endometrial tissues examined had no positive immunohistochemical staining for HIF-1α, including two full thickness hysterectomy specimens from the perimenstrual phase.

These studies consistently show that localisation of HIF-1α is maximal in glandular epithelial cells but results are conflicting in regard to the timing of its presence. This may be due to varied tissue collection techniques, antibody sensitivity and specificity or classification of the tissue samples studied. The poor quality immunohistochemical results detailed in this chapter, alongside the conflicting results in the published literature, suggest that immunohistochemistry is not the most robust method of assessing endometrial HIF-1α. Therefore, protocols for nuclear

protein extraction from frozen endometrial samples and Western blotting for HIF-1α were optimised. This revealed a tightly controlled temporal and spatial pattern of HIF-1α in the human endometrium, exclusively in the perimenstrual phase. The HIF-1β subunit is a constitutively expressed nuclear protein and has previously been detected in the human endometrium throughout the menstrual cycle (Critchley et al., 2006b).

5.4.2 Hypoxic repair factor induction

The contribution of HIF-1α to the hypoxic induction of endometrial repair factors was assessed using echinomycin, a small molecule that inhibits the DNA binding of HIF to the hypoxic response element sequence but does not affect NFκB binding (Kong et al., 2005). In addition, HIF-1α was silenced with RNA interference. Both of these methods significantly abrogated the hypoxic induction of IL-8, AM and VEGF, suggesting that hypoxia is dependent on the presence of active HIF-1α for the regulation of these factors. The Western blot findings detailed herein show that hypoxic conditions stabilise HIF-1α protein in endometrial cells and provide further support for the involvement of HIF-1α in hypoxic repair factor induction.

These findings concur with data from other tissue sites. A hypoxic response element is present in the VEGF promoter region and HIF-1 has been shown to regulate VEGF expression in hypoxia treated cells from the liver, pancreas and prostate (Forsythe et al., 1996, Gray et al., 2005). In contrast, HIF-1 independent hypoxic regulation of VEGF has been described in colon cancer cells, where NFkB is thought to play an active role in its regulation (Mizukami et al., 2004). The findings in this chapter confirm that hypoxic regulation of endometrial VEGF is mediated by HIF-1α. Hypoxia has also been shown to increase AM expression via HIF-1 in cardiomyocytes and human tumour cell lines (Cormier-Regard et al., 1998, Garayoa et al., 2000), consistent with findings herein. The hypoxic response element has also been previously identified in the IL-8 promoter (Kim et al., 2006). HIF-1 has been shown to up-regulate IL-8 mRNA in human fibroblast-like synoviocytes (Ahn et al., 2008). Our findings in endometrial cells revealed a significant reduction in hypoxia mediated IL-8 mRNA with pharmacological inhibition of HIF-1 binding. However,

silencing of HIF-1α revealed inconsistent results, with one construct against HIF-1α significantly abrogating the hypoxic induction of IL-8 and the other construct having no impact on IL-8 expression. In colon cancer cells, IL-8 was shown to have a complex regulation in hypoxic conditions, with compensatory pathways activated when HIF-1α was silenced (Mizukami et al., 2005). Therefore, in endometrial cells HIF-1α may be sufficient to induce IL-8 in hypoxia but may not be necessary.

5.4.3 Prostaglandin repair factor induction

The contribution of HIF-1 to prostaglandin induction of endometrial repair factor expression was also assessed. Firstly, endometrial cells were treated with PGE_2 in normoxic conditions to assess its effect on HIF-1α protein. Although 100nM PGE_2 significantly increased IL-8 expression in normoxic conditions, it did not induce HIF-1α protein at any of the time points examined. However, inhibition of HIF-1 binding using echinomycin significantly abrogated the PGE_2 induced IL-8 expression and $PGF_{2\alpha}$ induced VEGF mRNA. These results suggest that HIF-1α may play a role in the normoxic induction of repair factors. However, as well as inhibition of HIF-1 binding, echinomycin has also been shown to reduce c-Myc and AP-1 binding by 30% and 50% respectively (Vlaminck et al., 2007). Inhibition of these transcription factors may also contribute to the decreased IL-8 and VEGF mRNA observed when endometrial cells are co-treated with echinomycin and prostaglandins.

To specifically assess the contribution of HIF-1α, RNA interference experiments were carried out. In contract to echinomycin experiments, these data revealed that $PGF_{2\alpha}$ induced endometrial VEGF mRNA was independent of HIF-1α. A previous study of endometrial adenocarcinoma explants and cells showed that $PGF_{2\alpha}$ activates the extracellular signal-regulated kinase 1/2 signaling pathway in an epidermal growth factor receptor dependent manner to increase VEGF promoter activity (Sales et al., 2005). Further studies are required to determine if a similar mechanism of action is present in normal endometrial tissue. Similarly, AM mRNA up-regulation on exposure of endometrial cells to $PGF_{2\alpha}$ was also found to be independent of HIF-1α. These results in endometrial cells differ from those in preadipocytes, where

treatment with $PGF_{2\alpha}$ in normoxia resulted in increased HIF-1α protein due to increased HIF-1α transcription (Liu and Clipstone, 2008).

The contribution of HIF-1α to the up-regulation of IL-8 by PGE_2 was more difficult to define. Two ShRNA constructs against HIF-1α were used and neither resulted in a significant abrogation of PGE_2 induction of IL-8, although one construct did lead to a marked decrease in IL-8 expression. NFκB is a nuclear transcription factor present in the endometrium during the perimenstrual phase (King et al., 2001) and the NFκB binding site has been identified in the IL-8 promoter (Kunsch and Rosen, 1993). NFκB has been shown to up-regulate IL-8 mRNA expression in cells from other tissue sites in the body, even in the absence of HIF-1α (Mizukami et al., 2005). To assess the contribution of NFκB in both hypoxic and PGE_2 mediated endometrial IL-8 expression, an adenoviral dominant negative inhibitor of NFκB (Ad-IκBα) was utilised. This inhibitor maintains NFκB in a cytoplasmic location, preventing the transcription of its target genes. On infection of endometrial epithelial cells with Ad-IκBα, a significant decrease in PGE_2 mediated IL-8 mRNA up-regulation was demonstrated, with no significant decrease in hypoxic induction. Therefore, although both HIF-1α and NFκB may impact on endometrial IL-8 mRNA, HIF-1α appears to be dominant in hypoxic conditions and NFκB dominant in the regulation of IL-8 by PGE_2 in normoxia. Interactions between these two transcription factors may take place in the perimenstrual endometrium. There is mounting evidence for cross talk between NFκB and HIF-1 in other tissue sites (Walmsley et al., 2005, Frede et al., 2006, van Uden et al., 2008, Belaiba et al., 2007). Therefore, the presence of both of these transcription factors, and possible cross-talk between them, may explain the synergistic up-regulation of IL-8 mRNA seen in endometrial cells exposed to PGE_2 and hypoxic conditions simultaneously (Chapter 4.3.1.2).

These observations in human endometrial cells are not consistent with those in prostate cancer cells (Liu et al., 2002). In these cells, treatment with 1μM PGE_2 did significantly increase HIF-1α protein levels. A COX-2 inhibitor prevented this accumulation of nuclear HIF-1α in prostate cells in normoxic and hypoxic conditions. In colon cancer cells, PGE_2 also significantly increased HIF-1α protein,

and a SiRNA against HIF-1α abrogated the PGE_2 mediated VEGF mRNA increase (Fukuda et al., 2003). In contrast, the findings described in this chapter revealed that PGE_2 did not increase HIF-1α protein and indomethacin (a COX-2 inhibitor) had no effect on endometrial HIF-1α protein levels in hypoxia. These conflicting findings may be due to differences in cell type or may reflect the higher concentrations of prostaglandins used in *in vitro* cancer models, levels that would not be physiological in the endometrium. A study examining the induction of the angiogenic factor CYR61 in endometrial cells revealed by using SiRNA that its hypoxic induction was HIF-1α dependent (Gashaw et al., 2008). Interestingly, although this study showed that CYR61 was also up-regulated by PGE_2 and $PGF_{2\alpha,}$ it did not examine if these effects were HIF-1α mediated. Although HIF-1α has not been shown to have a significant role downstream of PGE_2 action in the endometrium, a recent study found that hypoxic induction of HIF-1α stimulated PGE_2 production, through stimulation of PGE synthase (Lee et al., 2010). Therefore, *in vivo* hypoxia in the perimenstrual endometrium may influence the synthesis of PGE_2, to enhance the induction of repair factors.

Whilst contributing to the existing literature on the role of HIF-1α in endometrial repair, these data also prompt further interesting questions. The presence of HIF-1α was examined in endometrial biopsies from across the cycle but HIF-2α was not studied. This alternative alpha subunit in the HIF-1 complex has a similar structure and regulation as HIF-1α but they appear to have differing transcriptional targets (Ratcliffe, 2007, Raval et al., 2005, Warnecke et al., 2004). Study of the functional responses to HIF-1α and HIF-2α across the genome revealed that, although HIF-2α binding affinity was similar to that of HIF-1α at some HRE target sites, it contributed very little to the overall HIF response (Mole et al., 2009). Delineation of the presence of HIF-2α across the menstrual cycle may provide insight into the functional impact of the endometrial hypoxic response.

The role of NFκB in the induction of IL-8 by PGE_2 was examined using a dominant negative inhibitor. Time restrictions meant the role of this transcription factor in $PGF_{2\alpha}$ mediated repair factor induction could not be assessed. For the same reason,

the contribution of HIF-1α and NFκB to the production of prostaglandins was not examined. The mechanisms involved in prostaglandin repair factor induction are also still to be determined in the normal endometrium. These areas provide a focus for future research.

5.4.4 Summary

The results detailed in this chapter confirm the presence of HIF-1α protein in the perimenstrual endometrium. Hypoxia appears to regulate the expression of endometrial repair factors via induction of HIF-1α. $PGF_{2\alpha}$ acts independently of HIF-1α to increase AM and VEGF mRNA. IL-8 appears to have a more complex regulation, with NFκB playing an active role in its induction by PGE_2 in normoxic conditions.

6. Differences in Menstrual Phase Endometrium from Women with Heavy and Normal Menstrual Blood Loss

6.1 Introduction

Historically women of reproductive age had high parity and long periods of lactation, therefore menses were relatively infrequent. In contrast, women living in developed countries today have easy access to contraception and may expect to menstruate over 400 times during their reproductive life span. Hence, the gynaecological complaint of heavy menstrual bleeding (HMB) has become increasingly common in modern society. Over 800 000 women seek treatment per year in the UK alone (NICE, 2007) and menstrual complaints constitute greater than 20% of all secondary referrals to gynaecology outpatient clinics (Santer et al., 2005).

As discussed in Chapter 1.6.1, the definition of HMB may be subjective or objective. In the clinical setting, the subjective definition of "excessive menstrual blood loss that interferes with a woman's physical, emotional, social and/or material quality of life" (NICE, 2007) is most appropriate. However, to permit research into the pathogenesis of HMB, an objective definition is required. Early studies of menstrual blood loss (MBL) demonstrated that 90% of women had a loss of less than 80ml per menses (Hallberg et al., 1966a, Hallberg et al., 1966b, Hallberg and Nilsson, 1964). Therefore, 80ml has been adopted as the higher limit of normal menstrual bleeding (NMB). Conversely, the objective definition of HMB is a monthly MBL of greater than 80ml.

HMB may be caused by anatomical anomalies such as fibroids, adenomyosis or malignancy. However, a significant proportion of women with HMB have no structural abnormalities on investigation. These women were previously categorised as having "dysfunctional uterine bleeding". More recently, these women have been subclassified as having (i) a coagulopathy, (ii) ovulatory dysfunction, (iii) an iatrogenic cause or (iv) an endometrial cause for their HMB (Munro, 2010, Munro et al., 2011). The precise local mechanisms that are aberrant in women with endometrial HMB remain undefined. A host of local disturbances have previously been identified in women with HMB (Marsh et al., 1997, Smith et al., 1981b, Malik et al., 2006, Hewett et al., 2002, Mints et al., 2007, Rae et al., 2009, Smith et al., 2007). These studies have focused on factors involved in inflammation, haemostasis,

vasoconstriction and angiogenesis (see chapter 1.6.3); processes known to be involved in endometrial shedding and repair. An unbiased comparison of gene expression in the endometrium of women with HMB and NMB may identify novel causal factors but such a comparison is unavailable in the published literature to date.

As detailed in Chapter 3, factors with a putative role in endometrial repair are significantly increased during the menstrual phase of the cycle. Therefore, the experiments detailed herein focus on menstrual endometrium from women with HMB and NMB. A whole genome array was performed, analysed and partially validated to provide an unbiased comparison of menstrual endometrial gene expression between women with HMB and normal controls. In addition, as Hypoxia Inducible Factor (HIF) has been identified as an endometrial transcription factor with a potential role in repair (Chapter 5), its endometrial expression was examined in women with HMB and NMB. HIF-1α and HIF-1β mRNA and protein levels were measured in the two groups to elicit any aberrations in women with HMB. The expression of vascular endothelial growth factor (VEGF) was also examined, as an example of a downstream target gene of HIF. Furthermore, the functional impact of endometrial production of HIF-1α was delineated using an *in vitro* angiogenic assay.

6.2 Methods

6.2.1 Tissue collection

Endometrial tissue samples (n=71) were collected with written consent and approval from the local ethical committee as described in chapter 2.1. These women also collected their menstrual blood loss over one menses (Figure 11), using methods described in chapter 2.2. Further details of the endometrial biopsies utilised in this chapter can be found in tables 10 and 11.

6.2.2 Objective measurement of menstrual blood loss

Objective quantification of menstrual blood loss was carried out for each participant using a modified alkaline-haematin method, described in detail in chapter 2.2.

6.2.3 Menstrual pictogram chart analysis

Menstrual pictograms (Appendix 4) were completed by 32 of the 71 women who collected their menstrual blood loss. Five charts were excluded as they were filled out incorrectly or were not complete. Charts were scored as detailed in Chapter 2.2.2 using the scoring system outlined in Appendix 5.

6.2.4 RNA preparation

RNA was extracted from all 71 endometrial biopsies, as described in chapter 2.4.

Table 10. Clinical details for participants with objectively measured blood loss. MBL: menstrual blood loss.

	n	*Mean age (years)*	*Age Range (years)*	*Parous (n, %)*	*Mean MBL (ml)*	*Range MBL (ml)*
MBL <80ml	36	40	22-48	83	40	8-78
MBL >80ml	35	43	26-50	88	194	81-774

Table 11. Circulating oestradiol and progesterone levels at time of biopsy in women with heavy and normal bleeding. NMB: normal menstrual bleeding (<80ml). HMB: heavy menstrual bleeding (>80ml).

Stage of cycle	*Blood loss*	*n*	*Oestradiol in pmol/litre Mean (range)*	*Progesterone in nmol/litre Mean (range)*
Proliferative	**NMB**	10	742 (178-1942)	4.4 (1.2-6.6)
	HMB	11	725 (152-2004)	6.0 (1.2-16.7)
Early Secretory	**NMB**	8	600 (282-919)	31.4 (21.9-46.0)
	HMB	11	597 (321-817)	31.0 (25.3-36.3)
Mid Secretory	**NMB**	5	436 (228-780)	47.1 (8.5-118.9)
	HMB	5	486 (253-863)	57.7 (19.0-124.8)
Late Secretory	**NMB**	6	183 (65-371)	10.9 (8.9-20.6)
	HMB	4	224 (71-316)	14.3 (3.89-26.5)
Menstrual	**NMB**	7	196 (95-392)	3.1 (1.2-5.2)
	HMB	4	103 (61-144)	7.4 (1.5-18.4)

6.2.5 Illumina gene expression profiling

To perform an unbiased comparison of menstrual endometrial gene expression in women with objectively measured heavy and normal blood loss, whole genome array analysis was carried out (Finnish DNA Microarray Centre (FDMC), Turku Centre for Biotechnology). RNA from eight endometrial biopsies from the menstrual phase (four HMB and four NMB) was sent to FDMC for comparison (Table 12). The RNA quality was assessed for each sample using the Agilent Bioanalyser and quantified using the Nanodrop spectrophotometer as described previously (section 2.4.1) (Table 12). RNA was diluted to a concentration of 150ng/μl with RNase free water and frozen at -80°C before packaging in 5kg of dry ice for transport to the FDMC.

Array analysis was carried out by the FDMC using the Illumina Human HT-12 v.3 Expression BeadChip. Each array on this chip targets more than 25 000 annotated genes with more than 48 000 probes and the expression value for each of the probe types is calculated from approximately 15 measurements. Amplification was carried out on 300ng total RNA with Ambion's Illumina RNA TotalPrep Amplification kit. The centre confirmed RNA/cRNA quality prior to and following amplification with the Nanodrop ND-1000 and experion electrophoresis station (BioRad).

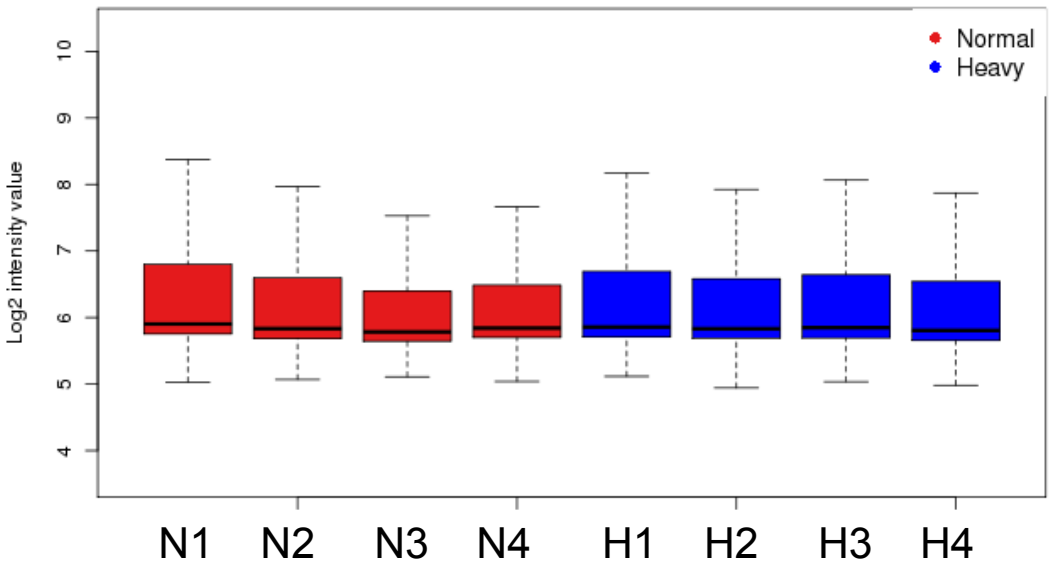
750ng of biotin labelled cRNA of each sample was hybridized to Illumina's Sentrix® HumanHT-12 Expression BeadChips, version 3 (cat no BD-103-0603) at 58 °C overnight (18 h) according to Illumina® Whole-Genome Gene Expression Direct Hybridization protocol, revision A. Hybridization was detected with 1 μg/ml Cyanine3-streptavidine (GE Healthcare Limited, UK). Chips were then scanned with Illumina BeadArray Reader, BeadScan software version 3.5. Numerical results were extracted with GenomeStudio2008v1. The data were normalised using the quantile normalisation method to remove variation between samples caused by non-biological sources (Figure 79). Biotin labelling was confirmed as homogenous for all samples.

Table 12. Classification of menstrual endometrial biopsies analysed using the Illumina Human HT v3 Expression Bead Chip. MBL: menstrual blood loss. RIN: RNA integrity number.

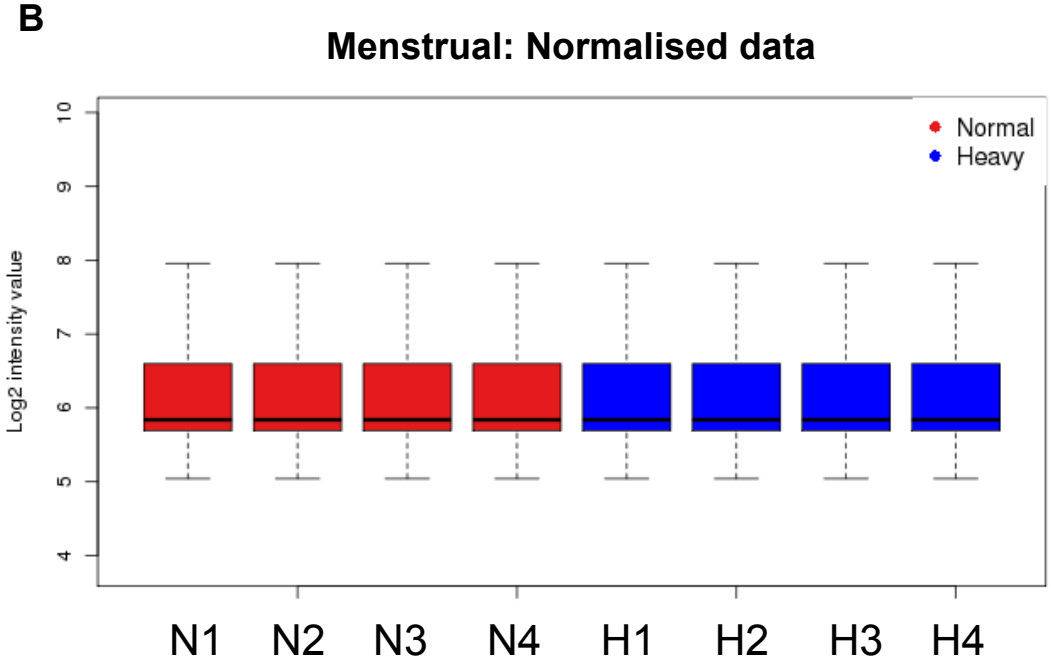
Histology	***Day of Cycle***	***Serum Oestradiol (pmol/litre)***	***Serum Progesterone (nmol/litre)***	***MBL (ml)***	***RIN***
Menstrual	1	226	2.17	26	9.7
Menstrual	7	95	1.24	33	10
Menstrual	2	187.2	5.15	39	10
Menstrual	2	108	3.16	42	10
Menstrual	3	77.4	6.84	84	9.6
Menstrual	4	60.6	2.61	91	9.9
Menstrual	4	129	1.53	277	10
Menstrual	2	144.3	18.44	287	9.8

Figure 79. **Box plots of expression intensity value distributions showing hybridisation of samples to Illumina array BeadChips.** (A) Log2-transformed signal intensity distributions of samples before normalisation. (B) Normalised data. N1-4: four samples from women with normal menstrual bleeding (<80ml). H1-4: four samples from women with heavy menstrual bleeding (>80ml).

A


Menstrual: Original data
Normal
Heavy
Log2 intensity value
4
5
6
7
8
9
10
N1
N2
N3
N4
H1
H2
H3
H4

B


Menstrual: Normalised data
Normal
Heavy
Log2 intensity value
4
5
6
7
8
9
10
N1
N2
N3
N4
H1
H2
H3
H4

Sample relations were examined by correlation and cluster analysis using Pearson's metrics and by Principal Component Analysis (PCA). Following the explorative analysis, a rigorous statistical approach was utilised to identify a subset of differentially expressed genes. R package LIMMA was used to perform statistical testing of changes in gene expression in endometrium from women with normal and heavy menstrual loss. Thresholds used for filtering the differentially expressed (DE) genes are shown in Table 13 and are based on the statistical significance (p-value) and the size of the difference in the mean expression levels between the groups (FC/logFC).

6.2.6 Functional "*in silico*" analysis

To obtain further insight into the functional and clinical significance of the differentially regulated genes identified by microarray analysis, the FDMC performed global testing using the globaltest package in R, utilising the gene ontology (GO) project. A known limitation of the GO project is that it assumes array targets are independent and relies on the hierarchical structure of the GO annotation. Therefore, with the help of Dr Elaine Marshall, further functional analysis was carried out using GeneGo, a commercial server data mining tool. This tool allows for the comparison of gene sets derived from experiments such as microarrays and predicts relationships between gene products based on peer-reviewed literature in the public domain. Functional GeneGo processes and functional gene networks were obtained by uploading differentially expressed gene lists to MetaCore™ version 5.4 build 19940 (GeneGo, Inc. MI, USA).

Table 13. Thresholds used in filtering differentially expressed transcripts and corresponding numbers of results. FC: fold change.

	FC	***log FC***	***P Type***	***P***	***Mean signal***	***Total***	***Up***	***Down***
Normal v Heavy	1.2	0.260	p-value	0.01	100	259	171	88

6.2.7 Validation of microarray data by Q-RT-PCR

To confirm the results of the statistical analysis and the validity of the gene list, Q-RT-PCR was performed on a subset of candidate genes. cDNA was prepared from RNA samples and Taqman Q-RT-PCR performed as previously described (Chapter 2.4). Menstrual endometrial biopsies from at least four different women were assessed in each blood loss group. PCR primers and probes used in this chapter are detailed in Chapter 2, Table 3. Levels of CXCR4, ESM1, HEY-1, SMAD-3, ACTG2, ESR1, PNKD and IDH1 mRNA expression were measured. These candidates were selected as they were identified as the most differentially expressed genes between the two groups. Levels of HIF-1α, HIF-1β and VEGF mRNA were also examined.

6.2.8 HIF-1α Western blot

Nuclear protein was extracted from eight menstrual phase endometrial biopsies (NMB n=4, HMB n=4) as described previously (chapter 2.5.2). HIF-1α protein was detected in these samples by Western blot (chapter 2.5.3 to 2.5.5) with β-actin used as an internal loading control. Densitometric analysis was carried out as described in chapter 2.5.6.

6.2.9 VEGF Immunohistochemistry

VEGF was immunolocalised in menstrual endometrial biopsies from women with objectively measured MBL using the protocol described in chapter 2.6.

6.2.10 VEGF ELISA

Four women in the menstrual phase with objectively measured blood loss (HMB n=2, and NMB n=2) provided enough tissue for *in vitro* culture. Tissue was weighed and incubated on a raised platform in 1ml of RPMI for 24h. Endometrial explant supernatants were pre-diluted to normalise for tissue weight. VEGF levels in the culture supernatant were then measured using a commercially available VEGF ELISA as previously described (chapter 2.7.3).

6.2.11 *In vitro* capillary tube formation assay

To assess the angiogenic impact of endometrial HIF-1α production we utilised an endometrial epithelial cell line (Ishikawa) and human umbilical vascular endothelial cells (HUVECs). HIF-1α was silenced in endometrial cells using lentiviral ShRNA constructs, as described in Chapter 2.9. Untransfected cells and cells transfected with HIF-1α/shRNA1470, HIF-1α/shRNA2192 or HIF-1α/shRNASCR were covered with 1ml of serum free DMEM media and placed in hypoxic conditions (0.5% O_2) for 8h. The conditioned media (CM) was harvested and stored at -20°C.

A HUVEC capillary tube formation assay was carried out and analysed as described in chapter 2.8. HUVECs were treated with 200µl of CM from (i) untransfected cells, (ii) HIF-1α/shRNA1470 transfected cells, (iii) HIF-1α/shRNA2192 transfected cells, (iv) HIF-1α/shRNASCR transfected cells (v) HIF-1α/shRNA1470 transfected cells plus 300ng/ml VEGF protein or (vi) HIF-1α/shRNA2192 transfected cells plus 300ng/ml VEGF protein. Assays were carried out in triplicate and with CM from three separate experiments.

6.2.12 Statistical analysis

Statistical analysis carried out by the FDMC on Illumina microarray data is discussed in section 6.2.5. For mRNA expression in endometrial tissue, results for each gene were expressed as a quantity relative to a comparator; a sample of RNA from the liver or placenta depending on gene expression. Tissue samples with a 2^ddCt value greater than two standard deviations from the mean of a group were defined as outliers and excluded from statistical analysis. Statistical differences in menstrual gene expression between women with HMB versus NMB were determined using a Mann Whitney non-parametric test. Statistical analyses of differences across the cycle between women with HMB and NMB were determined using a two-way ANOVA with Bonferroni post-test analysis. Differences in capillary formation between culture supernatants were assessed using one-way ANOVA, with Tukey's post test analysis. Comparison of VEGF ELISA results and Western blot densitometry was carried out using Mann-Whitney non-parametric tests.

6.3 Results

6.3.1 MBL chart analysis

Menstrual pictogram scores (n=32) ranged from 11ml to 145ml (mean 76.2 ml, median 72.5ml). The objective alkaline-haematin measurements ranged from 21ml to 227ml (mean 74.9 ml, median 62.5ml). There was no significant difference in mean blood loss value between these two methods (p=0.87).

Assessment of linear regression between the two sets of values revealed R squared = 0.4131 (Figure 80). The agreement between the scores from the menstrual pictogram and the modified alkaline haematin measurement is shown in Figure 81. The menstrual pictogram had a sensitivity of 80% and a specificity of 81% in diagnosing HMB (as defined by modified alkaline haematin method values of >80ml). The associated κ statistic for the comparison between the menstrual pictogram and the modified alkaline haematin method was 0.60.

Importantly, women with objectively measured normal menstrual bleeding had a median duration of bleeding of 4 days, which was significantly shorter than women with HMB, median 6 days ($p<0.01$) (Figure 82).

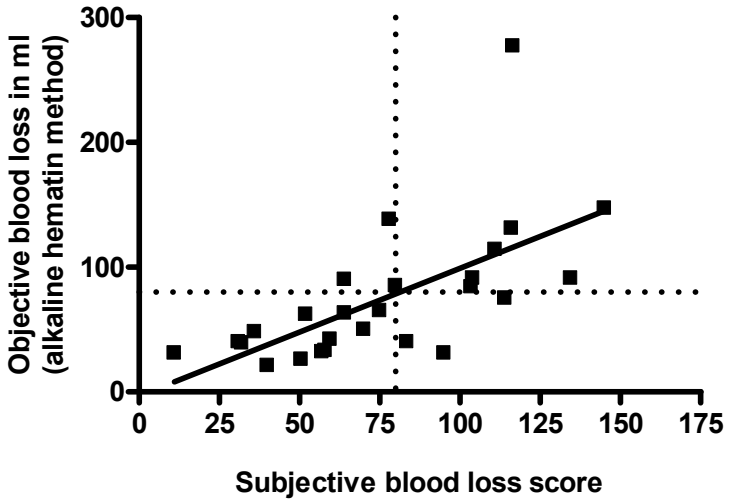


Figure 80. **Correlation between objective measurements of MBL by the modified alkaline haematin method and menstrual pictogram scores**. Dotted lines represent the arbitrary limit of 80ml by objective score (horizontal line) and pictogram score (vertical line).

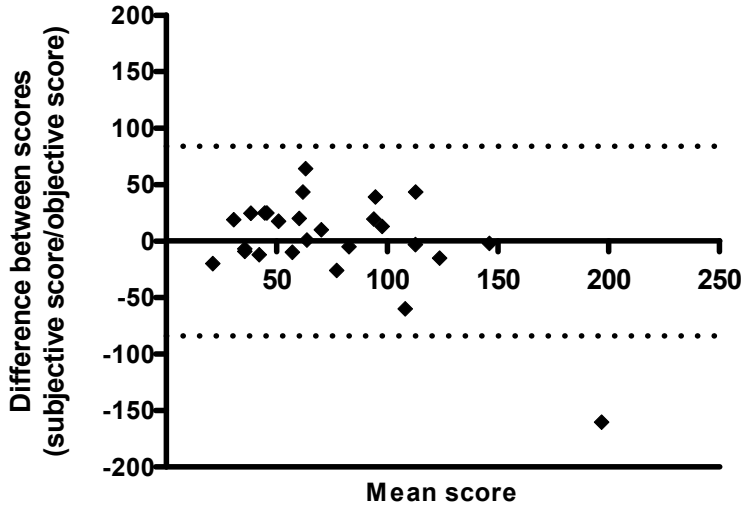


Figure 81. **Difference between menstrual pictogram and alkaline hematin blood loss measurement against mean score.** Horizontal dotted lines denote 2 SD.

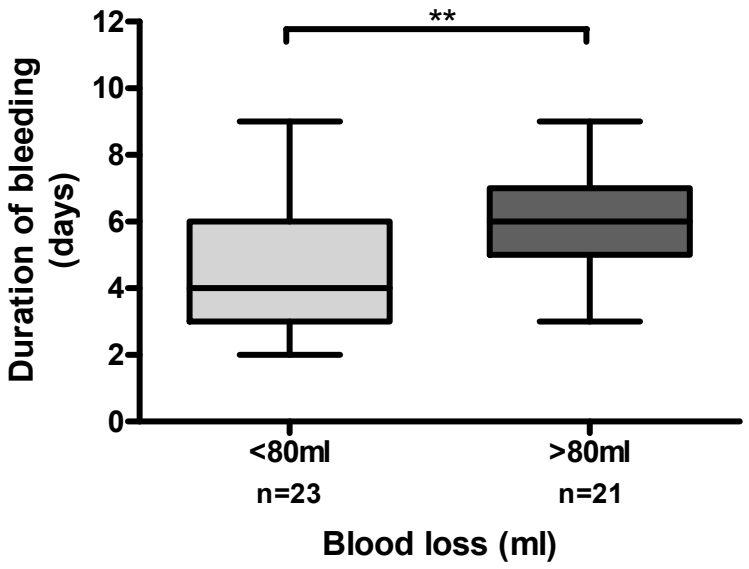


Figure 82. **Duration of menstrual bleeding in women with objectively measured normal (<80ml) and heavy (>80ml) blood loss**. **p<0.01.

6.3.2 Identification of differentially expressed genes in the endometrium of women with HMB

Analysis of the microarray dataset identified 259 transcripts displaying significant changes in the menstrual endometrium from women with heavy and normal menstrual blood loss ($p<0.01$) (Table 13). The relationship between the fold changes and average intensities of the filtered targets in the compared groups can be visualised in figures 83 and 84. There is no indication of an intensity dependency of log ratios. Of these differentially expressed (DE) targets, 171 transcripts were found to be up-regulated in the endometrium of women with HMB versus NMB and 88 transcripts were down-regulated (Table 14 and 15).

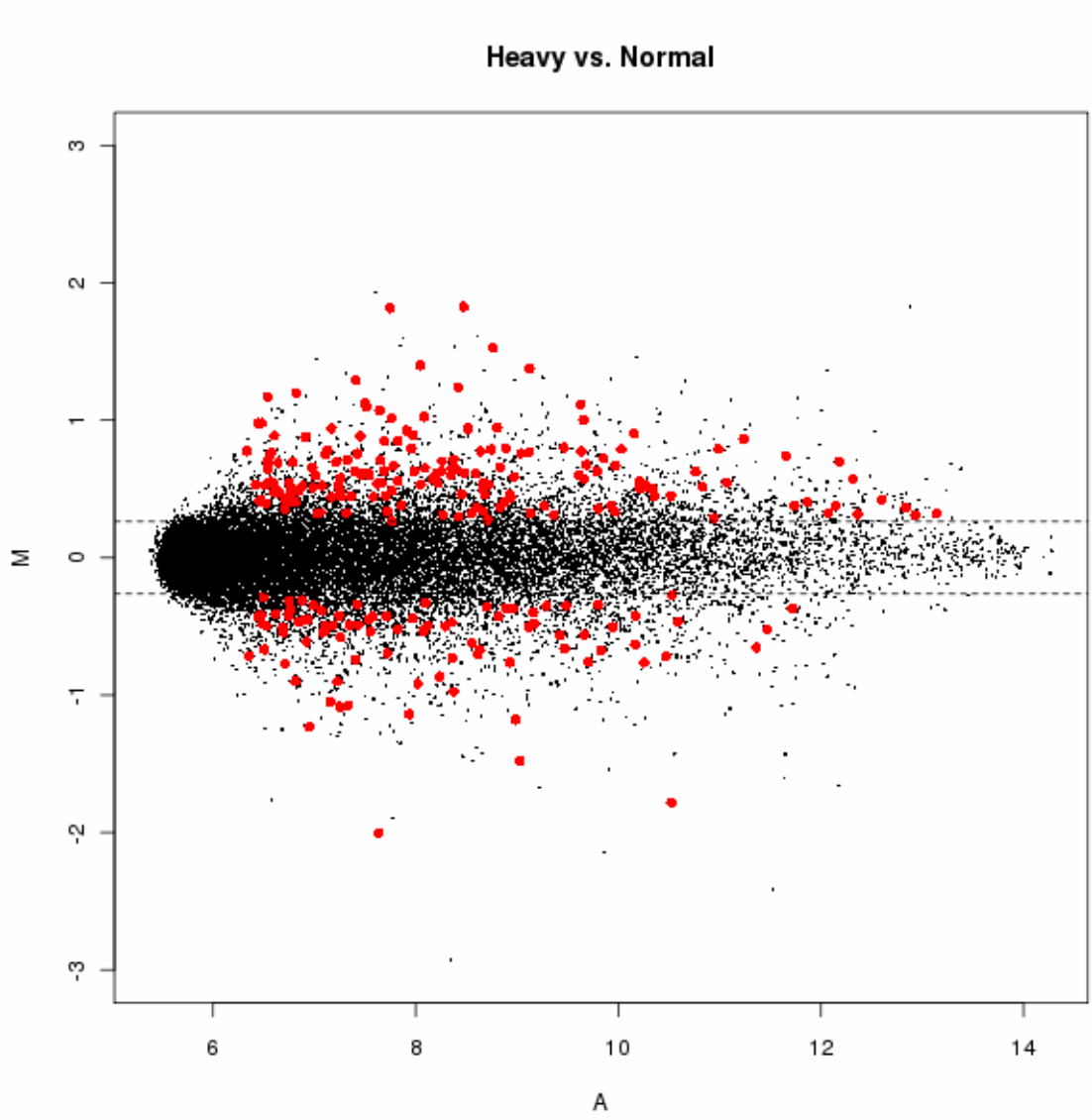


Figure 83. **MA plot.** The log2-transformed fold change (Y-axis) is plotted against the log2-transformed average intensity of the compared groups (X-axis). The differentially expressed targets are coloured red, the filtering threshold for log fold changed is marked with a dashed line.

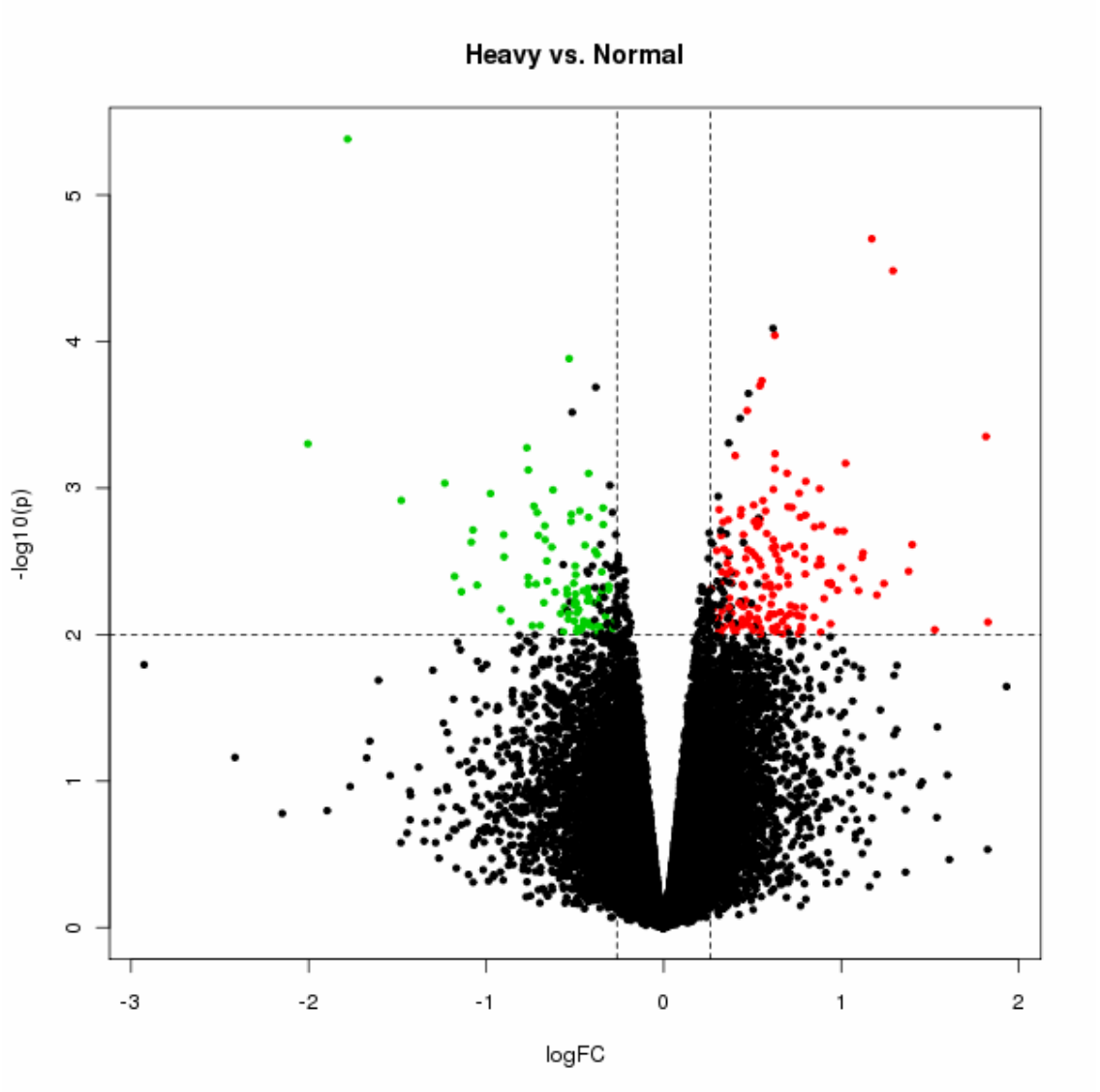


Figure 84. **Volcano plot**. The -log10 of the p-values (Y-axis) are plotted against the logFC (fold change) calculated for the normal versus heavy group (X-axis). Genes significantly expressed according to the t-test lie above the horizontal dashed line, genes with large FC lie outside the vertical lines. Red dots: up-regulated genes. Green dots: down-regulated genes.

Table 14. Transcripts identified by Illumina microarray analysis as being up-regulated in the menstrual endometrium of women with heavy menstrual bleeding versus normal controls.

Symbol	Source Reference ID	Mean HMB expression value	SD HMB	Mean NMB expression value	SD NMB	FC	logFC	P Value
ACTG2	NM_001615.3	434	172	117	27	3.518	1.815	0.000445
C15orf52	NM_207380.1	271	58	109	9	2.447	1.291	3.29E-05
FOLR1	NM_016731.2	141	23	62	6	2.253	1.172	1.99E-05
ATP6V0E2	NM_145230.2	451	144	170	57	2.638	1.399	0.002435
LRRC20	NM_018239.2	392	79	194	41	2.033	1.024	0.000678
MYH11	NM_002474.2	932	299	374	154	2.601	1.379	0.003705
NTHL1	NM_002528.4	281	96	123	18	2.177	1.122	0.002772
HLA-DQA1	XM_936128.2	1170	140	575	260	2.168	1.116	0.00298
MGC57346	XM_377476.4	318	92	154	30	2.018	1.013	0.001969
CD163	NM_203416.2	551	196	232	69	2.362	1.24	0.004486
FOLR1	NM_016724.1	127	42	62	8	1.971	0.979	0.001969
C10orf116	NM_006829.2	305	102	140	23	2.099	1.069	0.004134
CERCAM	NM_016174.3	167	42	89	12	1.838	0.878	0.001012
GINS2	NM_016095.1	182	77	77	26	2.298	1.201	0.005375
IDH1	NM_005896.2	1148	151	602	248	2	1	0.003487
COL24A1	NM_152890.4	278	91	130	44	2.139	1.097	0.00502
KDELR3	NM_006855.2	346	58	189	45	1.853	0.89	0.001803
RHOD	NM_014578.2	309	57	172	34	1.801	0.849	0.001845
	Hs.579631	813	526	199	67	3.545	1.826	0.008225
THRA	NM_003250.4	202	39	109	38	1.919	0.94	0.004425
GAMT	NM_000156.4	169	41	90	17	1.841	0.88	0.00305
CAPN13	NM_144575.2	133	17	75	27	1.848	0.886	0.003332
SLCO2B1	NM_007256.2	634	153	334	108	1.925	0.945	0.004579
GAMT	NM_000156.4	131	42	64	11	1.972	0.979	0.004986
OLFML3	NM_020190.2	333	41	182	56	1.899	0.925	0.004459
ETFB	NM_001014763.1	940	150	542	100	1.741	0.8	0.000901
COL5A2	NM_000393.3	3306	624	1838	526	1.822	0.865	0.003369
QDPR	NM_000320.1	627	61	367	89	1.74	0.799	0.001529
RPESP	NM_153225.2	858	555	269	92	2.881	1.526	0.009279
ANGPTL2	NM_012098.2	1597	408	858	231	1.87	0.903	0.005677
ALDH3B2	NM_001031615.1	126	31	73	5	1.697	0.763	0.001084
PDGFRA	NM_006206.3	1384	176	814	191	1.73	0.791	0.002515
C16orf35	NM_001039476.1	737	148	431	68	1.703	0.768	0.001583
HLA-DMB	NM_002118.3	2682	258	1591	442	1.73	0.791	0.003055
C6orf64	NM_018322.1	331	57	194	49	1.737	0.797	0.003866
TMEM14A	NM_014051.3	517	116	278	96	1.918	0.94	0.008455
LSM5	NM_012322.1	1205	216	725	93	1.65	0.723	0.001356
HCFC1R1	NM_001002018.1	124	31	73	10	1.672	0.741	0.002827
RNASEH2A	NM_006397.2	142	25	87	9	1.618	0.694	0.000793
HLA-DRA	NM_019111.3	5973	907	3680	591	1.625	0.7	0.001344
NUDT18	NM_024815.3	283	66	160	45	1.799	0.847	0.007618

CETN3	NM_004365.2	260	46	159	29	1.635	0.709	0.002482
KDELR3	NM_016657.1	569	82	339	102	1.726	0.788	0.006511
MCM2	NM_004526.2	246	73	133	42	1.846	0.884	0.009651
C1QA	NM_015991.1	186	37	111	37	1.718	0.781	0.00755
PCCB	NM_000532.3	1044	112	663	156	1.598	0.676	0.00257
CD163	NM_004244.4	120	25	74	13	1.622	0.698	0.004002
LOC642989	XM_926370.1	397	87	244	48	1.626	0.702	0.004525
LOC401115	XM_937968.2	4245	912	2546	566	1.67	0.74	0.006503
ZNF74	NM_003426.2	231	63	134	25	1.69	0.757	0.007444
REEP6	NM_138393.1	186	56	107	10	1.684	0.752	0.007281
UBE2L6	NM_004223.3	2161	102	1406	155	1.542	0.625	9.08E-05
MCM6	NM_005915.4	1069	309	621	148	1.71	0.774	0.008801
FANCG	NM_004629.1	210	17	137	20	1.544	0.627	0.000584
KCTD12	NM_138444.3	434	106	263	54	1.64	0.713	0.006332
KITLG	NM_000899.3	111	46	62	4	1.711	0.775	0.009092
C1orf54	NM_024579.2	1108	132	719	98	1.542	0.625	0.000738
THEM2	NM_018473.2	540	163	310	63	1.709	0.773	0.009355
FLJ10986	NM_018291.2	342	9	222	54	1.57	0.651	0.003123
C3orf14	NM_020685.3	414	62	266	54	1.571	0.651	0.003559
CYBB	NM_000397.2	159	17	103	22	1.573	0.654	0.003714
CDK5	NM_004935.2	210	57	127	24	1.638	0.712	0.007215
HRSP12	NM_005836.2	230	31	150	19	1.534	0.618	0.001021
CD74	NM_004355.2	1120	223	718	94	1.548	0.63	0.002825
BST2	NM_004335.2	320	59	205	18	1.545	0.628	0.0025
TP53I3	NM_147184.1	698	172	416	119	1.691	0.758	0.009878
LRRC20	NM_018239.2	117	15	76	17	1.567	0.648	0.004739
EEF2K	NM_013302.3	449	84	290	27	1.533	0.616	0.002253
RAD51AP1	NM_006479.3	128	17	81	25	1.615	0.691	0.007478
PPA2	NM_176866.2	481	65	316	58	1.528	0.612	0.002569
LPXN	NM_004811.1	579	98	370	78	1.577	0.657	0.007043
C16orf53	NM_024516.2	192	44	119	25	1.619	0.695	0.008993
WDR51A	NM_015426.2	284	69	176	26	1.59	0.669	0.008143
C2orf79	NM_001013663.1	188	24	126	18	1.495	0.58	0.002045
TMEM160	NM_017854.1	990	82	671	108	1.488	0.573	0.001435
GALM	NM_138801.1	231	47	150	23	1.53	0.614	0.005378
FASTK	NM_033015.2	406	65	268	54	1.521	0.605	0.004792
LSM4	NM_012321.2	979	191	641	85	1.514	0.599	0.004541
DNASE2	NM_001375.2	1272	144	824	272	1.591	0.67	0.009929
CNFN	NM_032488.2	161	34	105	7	1.511	0.596	0.004554
MCM4	NM_005914.2	388	114	245	15	1.542	0.625	0.007254
ANKRD33	NM_182608.2	429	77	278	68	1.557	0.639	0.00878
ME2	NM_002396.3	221	47	145	23	1.521	0.605	0.006205
TIMELESS	NM_003920.2	277	33	188	23	1.473	0.558	0.001216
RAD51L3	NM_002878.2	115	11	78	5	1.466	0.552	0.000185
LOC728635	XM_001131304.1	261	58	168	36	1.551	0.633	0.009558
HLA-DRA	NM_019111.3	6265	937	4224	685	1.487	0.572	0.00403
PSEN2	NM_012486.1	231	34	154	35	1.516	0.6	0.007222
COMMD9	NM_014186.2	361	32	248	17	1.455	0.541	0.000201
SDSL	NM_138432.2	355	63	238	35	1.485	0.571	0.004662

C1orf53	NM_001024594.1	231	72	146	5	1.534	0.617	0.009384
ASAP3	NM_017707.2	367	30	245	62	1.529	0.613	0.009051
APRT	NM_000485.2	496	60	341	44	1.457	0.543	0.001627
SFXN4	NM_213649.1	628	135	414	61	1.504	0.589	0.007706
CATSPER2P1	NR_002318.2	118	20	81	3	1.452	0.538	0.001758
SNRPF	NM_003095.2	2632	500	1776	79	1.461	0.547	0.003387
LSMD1	NM_032356.3	240	47	164	13	1.452	0.538	0.003093
C7orf50	NM_032350.4	1453	264	984	133	1.469	0.555	0.005633
TMEM118	NM_032814.2	110	12	77	9	1.441	0.527	0.001668
GLYCTK	NM_145262.2	162	14	114	18	1.439	0.525	0.001835
KIAA1324L	NM_152748.2	131	19	91	6	1.421	0.507	0.001306
PSEN2	NM_000447.1	152	10	107	16	1.424	0.51	0.001698
MRPL37	NM_016491.2	2197	323	1530	154	1.429	0.515	0.0029
RPP40	NM_006638.2	514	96	352	32	1.444	0.53	0.006603
RIMS3	NM_014747.2	132	26	90	13	1.46	0.546	0.009091
PCBD1	NM_001001939.1	322	49	224	44	1.447	0.533	0.008171
LSM2	NM_021177.3	1553	206	1097	108	1.41	0.496	0.00272
YIF1B	NM_033557.1	249	52	170	23	1.458	0.544	0.009937
C16orf75	NM_152308.1	180	11	127	26	1.439	0.525	0.007747
C12orf48	NM_017915.2	104	15	72	14	1.445	0.531	0.00886
C1orf85	NM_144580.1	1519	379	1035	48	1.439	0.525	0.0083
PNKD	NM_015488.4	143	18	100	19	1.438	0.524	0.008803
FLJ23584	NM_024588.3	117	12	84	3	1.385	0.47	0.000296
RASSF4	NM_032023.3	137	23	97	5	1.398	0.483	0.003649
GLE1	NM_001003722.1	484	56	346	62	1.409	0.495	0.006281
C17orf53	NM_024032.2	115	13	83	10	1.388	0.473	0.002626
TRAPPC2L	NM_016209.2	1440	293	1002	85	1.418	0.503	0.009441
C8orf59	NM_001099672.1	577	92	416	25	1.378	0.463	0.003012
MCM4	NM_182746.1	120	21	85	6	1.398	0.483	0.007662
PET112L	NM_004564.1	182	18	131	25	1.394	0.48	0.007236
C5orf39	NM_001014279.1	260	36	186	30	1.402	0.488	0.008724
SLC27A3	NM_024330.1	193	19	141	15	1.364	0.448	0.002083
ME2	NM_002396.3	412	34	302	51	1.376	0.46	0.005827
COQ3	NM_017421.3	129	22	93	10	1.384	0.469	0.008055
EIF3K	NM_013234.2	1727	239	1261	142	1.367	0.451	0.004685
TSPAN4	NM_001025238.1	572	38	424	47	1.353	0.436	0.001404
CCDC51	NM_024661.3	122	13	90	7	1.351	0.434	0.00153
ATP5J2	NM_001003714.1	1539	222	1127	110	1.361	0.445	0.004563
C10orf61	NM_001013840.1	245	30	179	25	1.367	0.451	0.006457
CKS1B	NM_001826.1	487	91	351	32	1.373	0.457	0.008485
AIFM1	NM_145813.1	180	5	134	24	1.36	0.443	0.006042
CYP2R1	NM_024514.4	169	23	124	13	1.357	0.441	0.006347
UBLCP1	NM_145049.1	225	32	165	20	1.359	0.442	0.007806
ACOT11	NM_147161.2	170	31	124	9	1.358	0.441	0.008663
UQCRQ	NM_014402.4	7269	1086	5386	458	1.342	0.424	0.006119
LSM1	NM_014462.1	4299	211	3257	251	1.322	0.403	0.000602
C14orf143	NM_145231.2	126	10	95	12	1.325	0.406	0.003821
EBI3	NM_005755.2	104	16	77	9	1.341	0.423	0.00863
GTPBP8	NM_014170.2	102	14	77	10	1.329	0.411	0.009038

PNPO	NM_018129.2	546	63	412	55	1.326	0.407	0.008473
TSPAN17	NM_130465.3	129	5	99	17	1.324	0.405	0.009659
LOC550112	XR_001037.1	106	4	81	13	1.314	0.394	0.009329
NDUFB2	NM_004546.2	3925	488	3006	172	1.3	0.378	0.004394
COX10	NM_001303.2	705	28	546	69	1.298	0.376	0.003663
CCBL2	NM_001008661.1	266	14	205	30	1.304	0.383	0.007151
MRPS11	NM_176805.1	1125	178	857	57	1.304	0.382	0.008656
HIST1H4C	NM_003542.3	8315	597	6463	606	1.288	0.366	0.002761
TMEM188	NM_153261.4	442	28	344	28	1.286	0.363	0.001647
C1orf91	NM_019118.2	120	12	93	9	1.293	0.371	0.005641
PSMB6	NM_002798.1	5160	491	3993	481	1.296	0.374	0.008102
BRP44	NM_015415.2	520	69	401	28	1.29	0.367	0.006519
MFSD1	NM_022736.1	1015	111	790	33	1.28	0.356	0.003263
ASTE1	NM_014065.2	118	10	93	8	1.277	0.353	0.003869
SLC37A4	NM_001467.4	456	16	362	37	1.266	0.34	0.002611
CKAP5	NM_001008938.1	1127	65	894	63	1.26	0.334	0.001711
MRPL11	NM_170738.1	238	29	187	13	1.265	0.339	0.008819
FAM104A	NM_032837.1	629	41	502	31	1.251	0.323	0.002111
FBXO22	NM_012170.2	179	14	143	10	1.253	0.326	0.003726
SLC25A14	NM_003951.2	149	8	119	14	1.255	0.328	0.006921
	Hs.489254	146	8	117	11	1.249	0.321	0.004518
NEDD8	NM_006156.1	10116	743	8103	749	1.25	0.322	0.00635
FAM10A4	NR_002183.1	735	35	593	33	1.241	0.312	0.001404
SERF2	NM_001018108.2	4821	337	3870	456	1.25	0.322	0.009521
MCEE	NM_032601.2	419	37	336	35	1.248	0.32	0.009916
CEP63	NM_025180.3	344	17	278	32	1.242	0.313	0.007647
NDUFB8	NM_005004.2	5902	623	4735	368	1.244	0.315	0.009037
HEATR1	NM_018072.4	383	31	310	10	1.23	0.299	0.002678
37500	NM_004404.3	8693	552	7028	749	1.241	0.311	0.009421
CCDC72	NM_015933.3	2184	221	1783	61	1.22	0.287	0.007651
DIABLO	NM_019887.3	462	27	383	30	1.21	0.275	0.006712
ZFP161	NM_003409.2	240	16	199	9	1.201	0.264	0.004916

Table 15. Transcripts identified by Illumina microarray analysis as being significantly down-regulated in the menstrual endometrium of women with heavy menstrual bleeding versus normal controls.

Symbol	Source Reference ID	Mean HMB expression value	SD HMB	Mean NMB expression value	SD NMB	FC	logFC	P Value
CXCR4	NM_003467.2	799	108	2776	624	-3.436	-1.781	4.15E-06
ESM1	NM_007036.3	100	16	445	231	-4.012	-2.004	0.000499
CXCR4	NM_001008540.1	323	95	920	328	-2.786	-1.478	0.001214
ATP6V1C2	NM_144583.3	82	18	196	61	-2.351	-1.233	0.000927
LOC732075	XM_001131797.1	113	24	242	71	-2.106	-1.075	0.001933
LOC653583	XM_928224.2	107	25	231	75	-2.12	-1.084	0.002343
HEY1	NM_001040708.1	244	68	469	63	-1.967	-0.976	0.001091
SMAD3	NM_005902.3	352	124	795	243	-2.263	-1.178	0.00401
SLC3A2	NM_001013251.1	175	61	372	86	-2.203	-1.14	0.005095
BDKRB2	NM_000623.2	100	17	218	90	-2.072	-1.051	0.0046
LOC440895	XM_290985.6	110	14	212	69	-1.868	-0.902	0.002083
CHRNA3	NM_000743.2	84	21	157	42	-1.865	-0.899	0.002951
DUSP16	NM_030640.1	80	4	139	30	-1.706	-0.771	0.000531
MOAP1	NM_022151.4	378	62	640	95	-1.698	-0.764	0.000753
EFNA1	NM_004428.2	194	54	370	112	-1.888	-0.917	0.006709
TBX2	NM_005994.3	257	30	431	86	-1.66	-0.731	0.001328
HSPA5	NM_005347.2	952	184	1622	355	-1.698	-0.764	0.004065
IL6R	NM_000565.2	64	8	107	22	-1.64	-0.713	0.00147
LIMS1	NM_004987.3	656	162	1096	187	-1.696	-0.762	0.004548
ZNF295	NM_001098402.1	226	36	427	147	-1.82	-0.864	0.008146
YPEL2	NM_001005404.3	312	67	503	62	-1.632	-0.706	0.0021
DHRS3	NM_004753.4	1116	167	1860	450	-1.645	-0.718	0.004514
DAAM1	NM_014992.1	318	43	509	98	-1.591	-0.67	0.001811
BMP6	NM_001718.4	73	13	116	18	-1.589	-0.668	0.002255
NXF1	NM_006362.4	573	116	894	105	-1.579	-0.659	0.003146
CRY1	NM_004075.2	308	60	468	22	-1.541	-0.624	0.00103
ETS2	NM_005239.4	2120	343	3355	638	-1.575	-0.655	0.004303
TOM1	NM_005488.1	924	78	1451	299	-1.548	-0.631	0.002528
ARSD	NM_001669.2	134	28	224	52	-1.67	-0.74	0.008708
DDX21	NM_004728.2	730	140	1171	246	-1.596	-0.675	0.00606
UGT2B7	XM_943434.1	171	48	270	38	-1.619	-0.695	0.008685
SOX7	NM_031439.2	99	18	152	26	-1.528	-0.612	0.005135
ZNF654	NM_018293.2	115	8	167	13	-1.447	-0.533	0.000131
	Hs.492187	126	10	191	40	-1.496	-0.581	0.007185
UFM1	NM_016617.1	676	99	998	172	-1.473	-0.559	0.006512
EIF4A3	NM_014740.2	2373	210	3423	486	-1.436	-0.522	0.001692
HIST2H2BE	NM_003528.2	112	12	166	31	-1.459	-0.545	0.004891
CRY1	NM_004075.2	190	21	273	33	-1.434	-0.521	0.001509
CLN8	NM_018941.3	86	11	127	24	-1.458	-0.544	0.005326
DPY19L1	NM_015283.1	576	127	843	124	-1.479	-0.565	0.009606

LSM12	NM_152344.2	159	37	226	8	-1.451	-0.537	0.007183
OTUD4	NM_199324.1	227	44	327	43	-1.452	-0.538	0.007866
C6orf106	NM_024294.2	87	8	124	21	-1.421	-0.507	0.004473
YWHAG	NM_012479.2	78	8	111	16	-1.413	-0.499	0.003402
PANX2	NM_052839.2	116	9	166	28	-1.41	-0.496	0.003907
ICA1	NM_004968.2	266	32	378	64	-1.414	-0.5	0.005918
C20orf111	NM_016470.6	835	127	1180	158	-1.417	-0.503	0.006288
NUP153	NM_005124.2	235	35	331	47	-1.412	-0.498	0.005286
ZNF562	NM_017656.2	279	22	389	47	-1.387	-0.472	0.001433
UBR7	NM_001100417.1	123	7	175	36	-1.407	-0.492	0.006162
ACSL3	NM_203372.1	472	78	668	99	-1.417	-0.503	0.008089
RIPK4	NM_020639.2	139	19	195	29	-1.404	-0.489	0.007121
KIF1B	NM_015074.2	490	57	686	108	-1.394	-0.479	0.006773
SENP2	NM_021627.2	147	16	208	40	-1.404	-0.49	0.009598
ANAPC1	NM_022662.2	76	6	108	23	-1.4	-0.485	0.009011
TMEM165	NM_018475.2	217	30	292	11	-1.36	-0.443	0.002458
	Hs.24119	103	15	141	12	-1.37	-0.454	0.005029
BCL2L1	NM_138578.1	99	16	136	17	-1.382	-0.467	0.009319
DDX39	NM_005804.2	1322	154	1832	292	-1.379	-0.464	0.009045
SH3RF1	NM_020870.3	131	12	176	11	-1.341	-0.424	0.000795
AP2A2	NM_012305.2	161	28	218	18	-1.368	-0.452	0.008047
BRWD1	NM_033656.2	93	9	125	11	-1.342	-0.424	0.001587
C14orf43	NM_194278.3	106	18	144	16	-1.365	-0.448	0.008516
PPFIBP1	NM_003622.2	165	20	223	26	-1.349	-0.431	0.005159
SEH1L	NM_001013437.1	182	23	245	26	-1.343	-0.426	0.00484
RPS6KA3	NM_004586.2	76	11	101	8	-1.346	-0.429	0.00589
ATP2A2	NM_001681.2	998	90	1344	186	-1.34	-0.422	0.005493
LOC730820	XM_001127763.1	392	60	525	58	-1.346	-0.429	0.008277
FAM13A1	NM_014883.2	77	4	104	19	-1.337	-0.419	0.008633
PREI3	NM_015387.2	86	15	113	4	-1.328	-0.409	0.008217
LHFPL2	NM_005779.1	503	73	660	53	-1.318	-0.398	0.006003
YWHAE	NM_006761.3	118	14	154	9	-1.308	-0.387	0.002687
KIT	NM_000222.1	94	10	125	17	-1.318	-0.399	0.008826
RAP2C	NM_021183.3	96	8	124	11	-1.297	-0.375	0.002846
CSDA	NM_003651.3	2981	332	3852	353	-1.294	-0.372	0.006111
POFUT2	NM_133635.4	420	59	540	36	-1.291	-0.368	0.008756
RAD23B	NM_002874.3	442	58	570	54	-1.292	-0.37	0.009791
P2RX4	NM_002560.2	370	48	472	23	-1.281	-0.357	0.005591
CRK	NM_016823.2	636	53	811	71	-1.274	-0.349	0.003729
DHX32	NM_018180.2	795	63	1006	38	-1.267	-0.342	0.001368
HINT3	NM_138571.3	153	11	193	13	-1.267	-0.341	0.001775
CPEB3	NM_014912.3	113	8	144	15	-1.273	-0.348	0.005053
PAN3	NM_175854.5	560	84	708	18	-1.275	-0.35	0.008877
PRPF40A	NM_017892.3	244	13	308	37	-1.255	-0.327	0.007518
TAF5L	NM_014409.3	97	6	120	10	-1.24	-0.31	0.004639
CDCA4	NM_017955.2	106	5	131	12	-1.239	-0.309	0.004949
MDK	NM_001012334.1	82	5	100	10	-1.22	-0.286	0.00905
CTTN	NM_005231.2	1350	118	1626	81	-1.207	-0.272	0.008001

6.3.3 Cluster analysis

The DE transcripts from all patients were evaluated further with blind explorative hierarchical clustering using Pearson's metrics. Principle component analysis (PCA) depicts the variance in gene expression profiles in samples (Figure 85). On the three dimensional graph, the distance between two points is proportional to the degree of similarity of gene expression between them. The samples clustered into four groups, two groups from women with HMB and two with NMB. The heatmap of sample clustering (Figure 86) confirms that the samples demonstrate segregation into normal and heavy bleeding clusters.

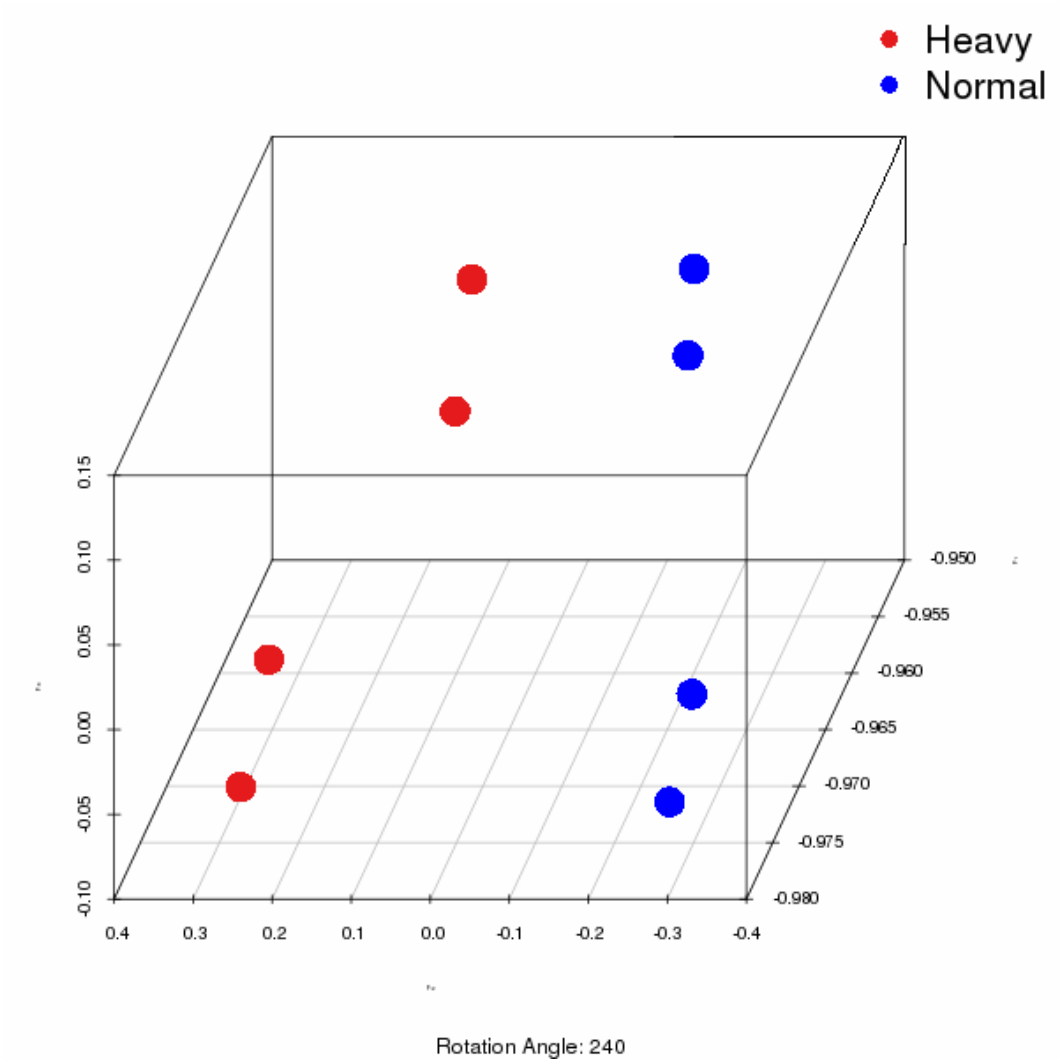


Figure 85. **Principle component analysis (PCA).** PCA was applied to all 8 endometrial samples that were characterised by gene expression on the Human TR-12 v3 Expression BeadChip and confirms sample segregation based on the statistics applied. X-axis: PCA component 1, Y-axis: PCA component 2, Z-axis: PCA component 3.

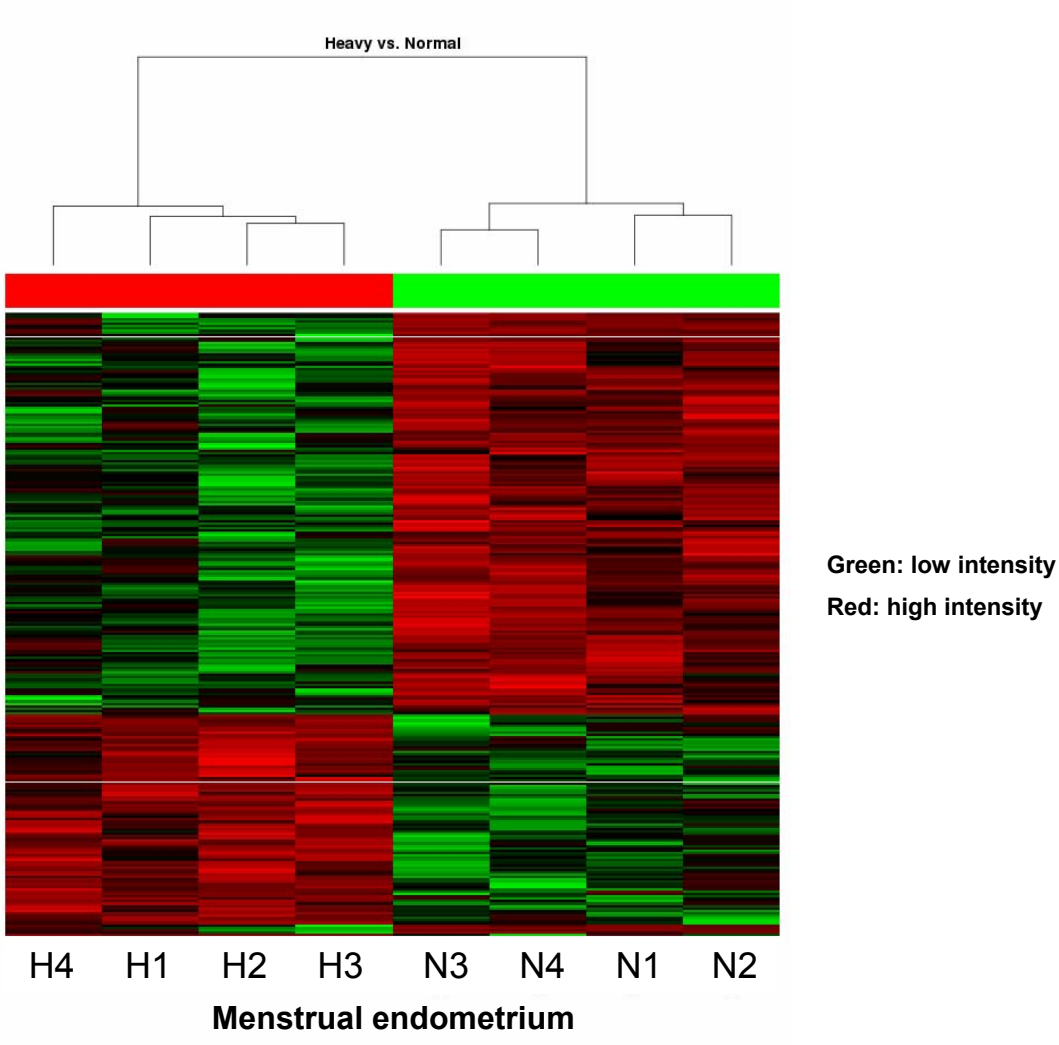


Figure 86. **Heat map.** Red represents high intensity and green low intensity. Samples included in each comparison at a time have been hierarchically clustered for the differentially expressed array targets. Each row in the plot represents one array target and they are organised by intensity measurement similarity.

6.3.4 Global test analysis

Statistical testing for over-representation of GO terms using globaltest package R revealed four categories with a p value of less than 0.05 (Table 16). Interestingly, the response to hypoxia was included in the top 10 global test GO results, although this category did not reach statistical significance. On examination of the 72 genes from the differentially expressed gene list that were targets for this term, there was evidence of a higher expression of genes known to have a role in the response to hypoxia in the endometrium of women with NMB than in those with HMB (Figure 87). These included CXCR4, SMAD3, EPAS, PLOD2, CITED2, THBS1 and HSP90B1.

Table 16. Top 10 globaltest GO results

Pathway	***P value***	***Genes***	***Description***
GO: 0000184	0.016	20	Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay
GO:0002026	0.019	10	Regulation of the force of heart contraction
GO:0000387	0.035	24	Spliceosomal snRNP biogenesis
GO:0000209	0.042	14	Protein polyubiquitination
GO:0000045	0.064	8	Autophagic vacuole formation
GO:0001710	0.066	7	Mesodermal cell fate commitment
GO:0001666	0.077	72	Response to hypoxia
GO:0000956	0.080	23	Nuclear-transcribed mRNA catabolic process
GO:0000578	0.081	7	Embryonic axis specification
GO:0001654	0.084	89	Eye development

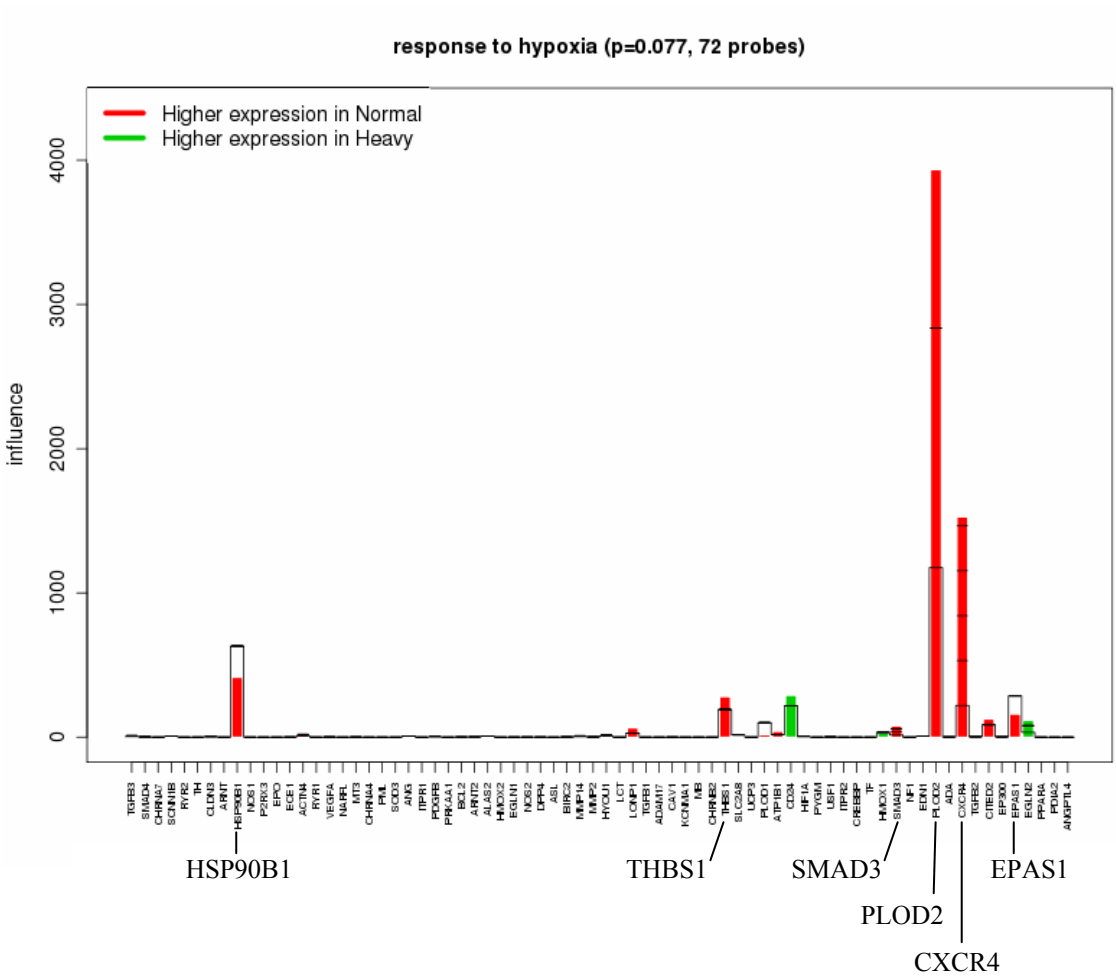


Figure 87. **Global test result for the 7th most significant GO pathway as a Gene Plot.** Red: higher expression in women with normal menstrual bleeding (<80ml). Green: higher expression in women with heavy menstrual bleeding (>80ml).

6.3.5 GeneGO analysis

To further define the functional differences in gene expression in the menstrual endometrium of women with NMB and HMB, GeneGO analysis of the differentially expressed gene set was carried out with the help of Dr Elaine Marshall. This analysis employs evidence-based interactions published in the literature and predicts functional networks and processes. Many of the bioprocesses identified were concerned with physiological events associated with repair, such as positive regulation of biological and cellular processes, leukocyte differentiation, regulation of apoptosis and response to stress/hypoxia. This finding of a pattern of consistent dysregulation among multiple genes in a pathway or network improves the validity of the microarray results.

A summary of the top ten GeneGo process networks is detailed in Figure 88. These are compiled by mapping the differentially expressed gene set onto approximately 110 cellular and molecular processes whose content is defined and annotated by GeneGo. Each process represents a pre-set network of protein interactions characteristic for the process. The most relevant network processes were determined using the parameters of (1) relative enrichment with the uploaded data and (2) relative saturation of networks with canonical pathways and are detailed in Table 17.

Further analysis using MetaCore^{TM} network building algorithms revealed 30 sub-networks (Table 17) that were ranked according to p-value (calculated using the basic formula for hypergenometric distribution), z-score (rank of sub-network according to saturation with the objects from the differentially expressed gene list) and g-score (modification of the z-score based on the number of canonical pathways used to build the sub-network). The network diagrams for the 2nd, 3rd and 24th ranked sub-networks are shown in Figures 89, 90 and 91. These diagrams detail the interactions between genes involved in a biological process, including their up-stream and down-stream targets. Differentially expressed genes in the comparison of menstrual endometrium from women with HMB and NMB are marked with a blue circle (down regulated in HMB) or red circle (up regulated in HMB).

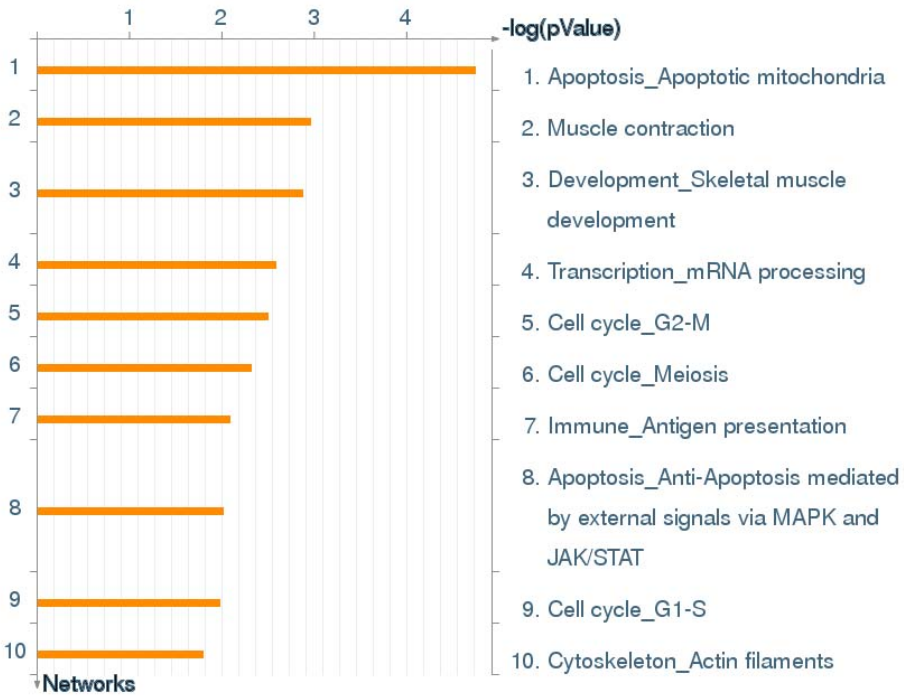


Figure 88. **Top ten GeneGo process networks.**

Table 17. Most Relevant GeneGo Networks

No	Processes	Size	Target	Pathways	p-Value	zScore	gScore
1	Response to steroid hormone stimulus (20.9%), interspecies interaction between organisms (20.9%), translation (20.9%)	50	19	6	6.09e-34	43.77	51.27
2	DNA metabolic process (32.4%), recombinational repair (11.8%), double-strand break repair via homologous recombination (11.8%)	50	19	0	7.08e-35	45.80	45.80
3	Regulation of cell cycle (35.6%), cell cycle process (35.6%), cell cycle (40.0%)	50	14	4	4.47e-23	32.14	37.14
4	Neuron differentiation (22.7%), cell communication (59.1%), system development (45.5%)	50	16	0	8.72e-27	35.64	35.64
5	Transmembrane receptor protein tyrosine kinase signalling pathway (40.8%), enzyme linked receptor protein signalling pathway (40.8%), cell communication (83.7%)	50	8	8	2.42e-11	17.60	27.60

Table 18.The most relevant sub-networks, compiled using GeneGo functional analysis of the differential expressed gene set from the comparison of menstrual endometrium from women with normal and heavy menstrual bleeding. Sub-networks subjected to further analysis in this chapter are highlighted in yellow.

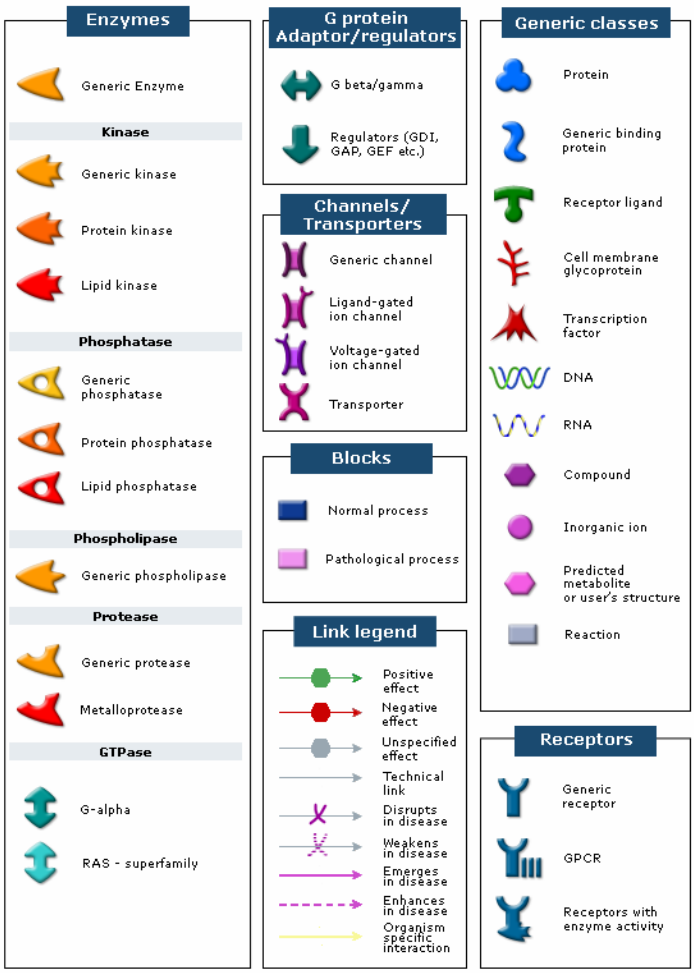
No	Key network objects	GO Processes	Total nodes	Root nodes	Pathways	p-Value	zScore	gScore
1	PNPO, DDX48, NXF1, APRT, MCM4	cellular nitrogen compound metabolic process (66.7%), nitrogen compound metabolic process (66.7%), nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (61.5%), mRNA transport (15.4%), establishment of RNA localization (15.4%)	50	18	0	3.49e-33	45.13	45.13
2	SMAD3, c-Kit, DDEFL1 (ACAP4), MGF, DDX21	regulation of signal transduction (34.0%), regulation of signalling process (34.0%), response to organic substance (36.2%), positive regulation of cellular process (46.8%), regulation of multicellular organismal process (36.2%)	50	15	6	3.27e-25	34.75	42.25
3	SMAD3, HLA-DRA1, ACTG2, MYH11, CCBL2	regulation of biological quality (48.7%), regulation of multicellular organismal process (41.0%), regulation of anatomical structure size (25.6%), biological regulation (87.2%), regulation of system process (25.6%)	50	15	2	3.41e-26	37.10	39.60
4	SMAD3, TBX2, HEY1, TR-alpha, CRK	positive regulation of biological process (72.0%), positive regulation of cellular process (68.0%), positive regulation of macromolecule biosynthetic process (44.0%), positive regulation of cellular biosynthetic process (44.0%), positive regulation of biosynthetic process (44.0%)	50	14	0	4.00e-23	32.41	32.41
5	CSDA, MHC class II, HLA-DM, CKS1, TBX2	positive regulation of biological process (68.0%), positive regulation of cellular process (64.0%), positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (44.0%), positive regulation of nitrogen compound metabolic process (44.0%), positive regulation of macromolecule metabolic process (48.0%)	50	14	0	4.00e-23	32.41	32.41
6	CSDA, PDGF-R-alpha, SOX7, GRP78, TR-alpha	signalling (65.2%), regulation of cell communication (39.1%), anatomical structure morphogenesis (39.1%), signalling process (56.5%), signal transmission (56.5%)	50	13	0	1.76e-21	31.03	31.03

No	Key network objects	GO Processes	Total nodes	Root nodes	Pathways	p-Value	zScore	gScore
7	SLC27A3, GAMT, DHRS3, OATP-B, FBX22	small molecule metabolic process (55.6%), creatine biosynthetic process (11.1%), cellular amino acid and derivative metabolic process (33.3%), amine metabolic process (33.3%), phosphagen biosynthetic process (11.1%)	50	10	0	2.87e-16	28.96	28.96
8	DDX21, RAD23B, MCM2, NTH1, RNASEH2A	DNA metabolic process (38.5%), macromolecule metabolic process (84.6%), primary metabolic process (89.7%), cellular macromolecule metabolic process (79.5%), cellular nitrogen compound metabolic process (66.7%)	50	12	0	1.42e-19	28.93	28.93
9	MHC class II, NUP153, SENP2, UBCH8, HLA-DRA1	multi-organism process (36.4%), positive regulation of biological process (52.3%), T cell differentiation (13.6%), lymphocyte differentiation (15.9%), regulation of phosphorylation (25.0%)	50	12	0	3.33e-19	28.01	28.01
10	Cortactin, ZF5, c-Kit, CRK, ESM-1	positive regulation of cellular process (59.2%), positive regulation of biological process (59.2%), biological regulation (87.8%), regulation of cellular process (83.7%), regulation of biological process (83.7%)	50	12	0	4.37e-19	27.72	27.72
11	CRY1, Midkine, AL3B2, NCU-G1 (C1orf85), TIM	circadian rhythm (23.7%), rhythmic process (26.3%), regulation of gene-specific transcription from RNA polymerase II promoter (21.1%), negative regulation of cellular process (50.0%), primary metabolic process (84.2%)	50	11	0	1.07e-17	26.79	26.79
12	Smac/Diablo, CRY1, Cortactin, Aif, TR-alpha	regulation of apoptosis (47.9%), regulation of programmed cell death (47.9%), regulation of cell death (47.9%), regulation of cellular process (91.7%), positive regulation of biological process (60.4%)	50	11	0	3.88e-17	25.37	25.37
13	ZF5, TBX2, P2X4, CRY1, Midkine	negative regulation of biological process (51.2%), negative regulation of cellular process (46.3%), cellular nitrogen compound metabolic process (58.5%), nitrogen compound metabolic process (58.5%), multicellular organismal process (65.9%)	50	11	0	1.56e-15	23.78	23.78

No	Key network objects	GO Processes	Total nodes	Root nodes	Pathways	p-Value	zScore	gScore
14	14-3-3 epsilon, CD163, CSDA, PSP, P2X4	response to stimulus (72.5%), biological regulation (90.0%), response to abiotic stimulus (30.0%), regulation of cellular process (85.0%), regulation of biological process (85.0%)	50	9	0	1.17e-13	21.36	21.36
15	Smac/Diablo, 14-3-3 epsilon, 14-3-3 gamma, CRK, Presenilin 2	regulation of apoptosis (63.3%), regulation of programmed cell death (63.3%), regulation of cell death (63.3%), positive regulation of cellular process (71.4%), positive regulation of biological process (71.4%)	50	9	0	2.13e-13	20.68	20.68
16	Cortactin, SH3RF, ETS2, CRK, CKS1	regulation of cellular process (94.0%), positive regulation of cellular process (62.0%), regulation of molecular function (48.0%), biological regulation (96.0%), regulation of biological process (94.0%)	50	9	0	2.13e-13	20.68	20.68
17	SLC3A2, Ephrin-A1, BDKRB2, CD74, MCM6	intracellular signalling pathway (40.0%), enzyme linked receptor protein signalling pathway (28.0%), transmembrane receptor protein tyrosine kinase signaling pathway (24.0%), protein amino acid phosphorylation (32.0%), signalling (66.0%)	50	9	0	2.13e-13	20.68	20.68
18	CD74, PDGF-R-alpha, SLC3A2, Ephrin-A1, MGF	negative regulation of apoptosis (39.4%), negative regulation of programmed cell death (39.4%), negative regulation of cell death (39.4%), regulation of apoptosis (45.5%), regulation of programmed cell death (45.5%)	50	8	0	4.82e-11	19.26	19.26
19	MKP-7, gp91-phox, P2X4, C16orf75, Ca-ATPase2	DNA metabolic process (35.5%), DNA repair (29.0%), cellular response to stimulus (45.2%), cellular response to stress (35.5%), response to DNA damage stimulus (29.0%)	50	8	0	1.09e-11	18.53	18.53
20	SMAD3, HEY1, NEDD8, PIG3, BDKRB2	positive regulation of biological process (70.8%), regulation of apoptosis (50.0%), positive regulation of cellular process (66.7%), regulation of programmed cell death (50.0%), regulation of cell death (50.0%)	50	8	0	1.30e-11	18.34	18.34

No	Key network objects	GO Processes	Total nodes	Root nodes	Pathways	p-Value	zScore	gScore
21	c-Kit, PDGF-R-alpha, Presenilin 2, TR-alpha, 14-3-3 epsilon	positive regulation of biological process (71.4%), positive regulation of cellular process (67.3%), regulation of cell communication (53.1%), regulation of signal transduction (49.0%), regulation of signalling process (49.0%)	50	8	0	1.30e-11	18.34	18.34
22	BDKRB2, c-Kit, Cortactin, SH3RF, PDGF-R-alpha	positive regulation of biological process (73.5%), positive regulation of cellular process (69.4%), regulation of programmed cell death (51.0%), regulation of cell death (51.0%), regulation of cell communication (55.1%)	50	8	0	1.30e-11	18.34	18.34
23	Presenilin 2, ETS2, PIR51, CSDA, PINCH	positive regulation of biological process (63.8%), positive regulation of cellular process (57.4%), regulation of biological process (91.5%), regulation of metabolic process (70.2%), regulation of cellular process (89.4%)	50	8	0	1.30e-11	18.34	18.34
24	SMAD3, ETS2, CSDA, CXCR4, CKS1	positive regulation of biological process (83.3%), positive regulation of cellular process (79.2%), regulation of programmed cell death (58.3%), regulation of cell death (58.3%), regulation of apoptosis (56.2%)	50	8	0	1.30e-11	18.34	18.34
25	CXCR4, SMAD3, c-Kit, CRK, TR-alpha	positive regulation of cellular process (69.4%), regulation of cell communication (57.1%), positive regulation of biological process (69.4%), cell differentiation (61.2%), leukocyte differentiation (28.6%)	50	7	0	6.84e-10	15.99	15.99
26	DHPR, Septin 2, Ca-ATPase2, SENP2, NEDD8	dihydrobiopterin metabolic process (8.7%), regulation of microtubule depolymerization (13.0%), negative regulation of microtubule depolymerization (13.0%), regeneration (21.7%), cellular aromatic compound metabolic process (21.7%)	50	5	1	2.13e-07	13.46	14.71
27	ME2, MCEE, Histone H4, p300, Succinyl-CoA	response to stress (55.6%), response to hypoxia (22.2%), cellular metabolic process (83.3%), cellular process (100.0%), response to oxygen levels (22.2%)	50	3	0	1.47e-04	9.08	9.08

No	Key network objects	GO Processes	Total nodes	Root nodes	Pathways	p-Value	zScore	gScore
28	CXCR4, CRK, SMAD3, BDKRB2, FAK1	intracellular signaling pathway (70.0%), protein amino acid phosphorylation (60.0%), phosphorylation (60.0%), positive regulation of cellular process (80.0%), positive regulation of biological process (82.0%)	50	4	0	3.49e-05	8.95	8.95
29	Presenilin 2, CSDA, ESR1 (nuclear), NF-kB, p300	regulation of apoptosis (63.3%), regulation of programmed cell death (63.3%), regulation of cell death (63.3%), positive regulation of biological process (79.6%), positive regulation of cellular process (73.5%)	50	2	0	1.45e-02	4.26	4.26
30	14-3-3 gamma, CRK, EGFR, p53, c-Src	regulation of programmed cell death (64.0%), regulation of cell death (64.0%), regulation of apoptosis (62.0%), response to stimulus (92.0%), positive regulation of cellular process (76.0%)	50	2	0	1.45e-02	4.26	4.26



Key for GeneGo subnetwork diagrams.

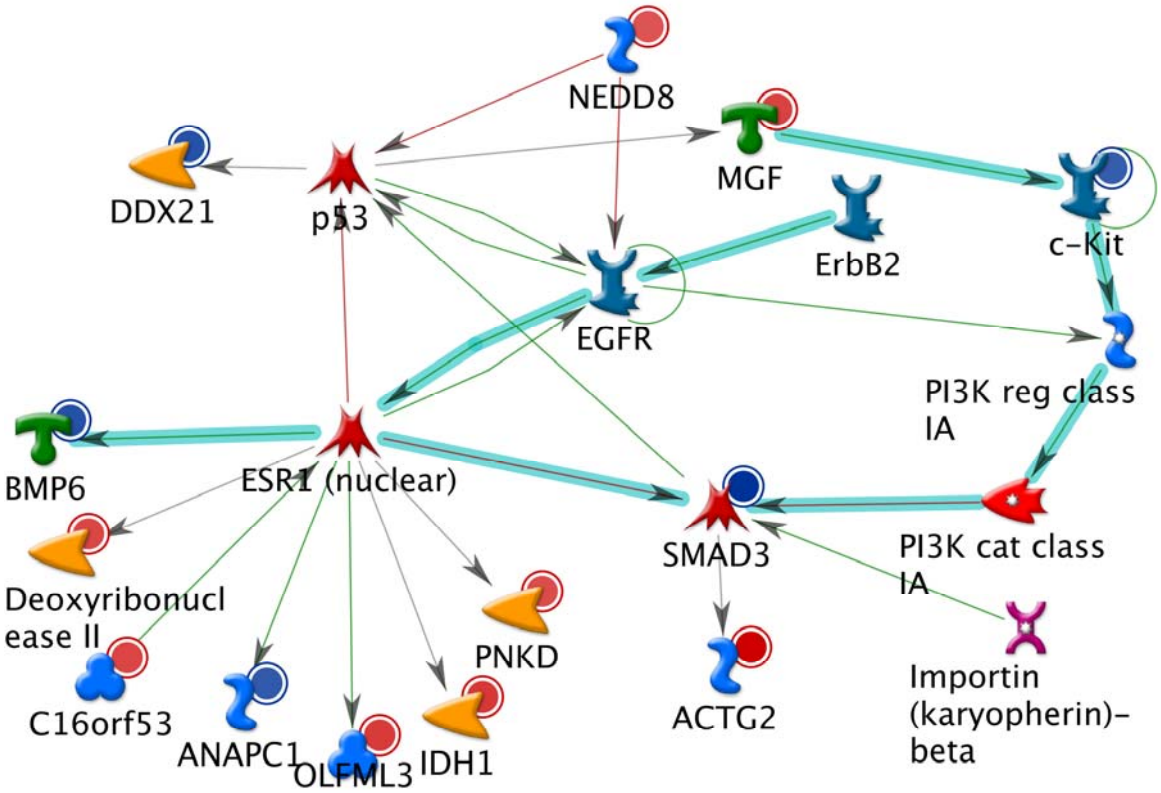


Figure 89. **Subnetwork 2 (Table 18)** detailing the interactions between genes involved in regulation of signal transduction, regulation of signalling process, response to organic substance, positive regulation of cellular process and regulation of multicellular organismal process. Red circles indicate genes up-regulated and blue circles genes down-regulated in menstrual endometrium from women with heavy menstrual bleeding versus normal controls. See key on page 290.

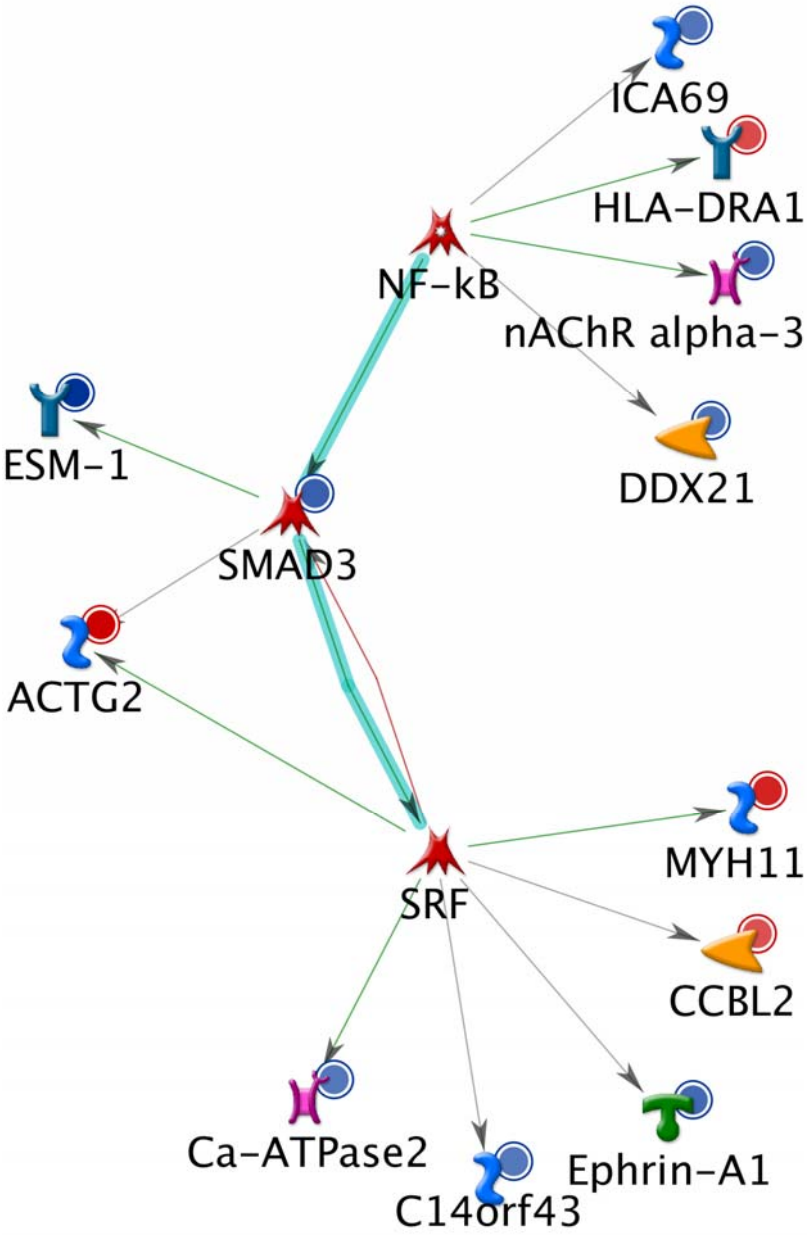


Figure 90. **Subnetwork 3 (Table 18)** detailing the interactions between genes involved in regulation of biological quality, regulation of multicellular organismal process, regulation of anatomical structure size, biological regulation and regulation of system process. Red circles indicate genes up-regulated and blue circles genes down-regulated in menstrual endometrium from women with heavy menstrual bleeding versus normal controls. See key on page 290.

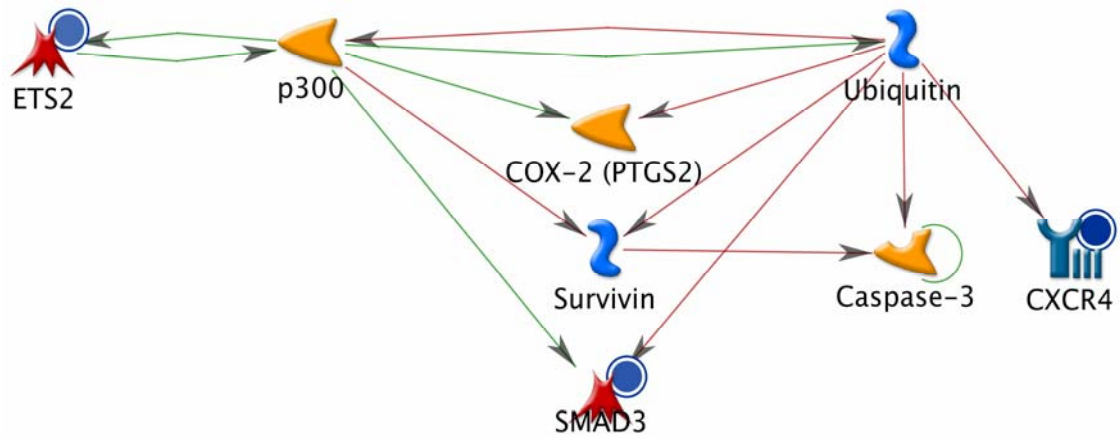


Figure 91. **Subnetwork 24 (Table 18)** detailing the interactions between genes involved in positive regulation of biological process, positive regulation of cellular process, regulation of programmed cell death, regulation of cell death and regulation of apoptosis. Blue circles indicate genes down-regulated in menstrual endometrium from women with heavy menstrual bleeding versus normal controls. See key on page 290.

6.3.6 Q-RT-PCR validation of microarray data

Four up-regulated and three down-regulated genes that showed statistical significance in the microarray dataset were selected for validation by Q-RT-PCR (Figures 92-98). These genes were selected as they showed the greatest fold changes of the differentially expressed dataset (Tables 14, 15) and appeared in functional subnetworks (Figures 89-91). Five out of the seven selected genes were confirmed to demonstrate statistically significant regulation in the same direction as microarray data (71% concordance). Endothelial cell-specific molecule 1 (ESM-1), chemokine receptor 4 (CXCR4) and SMAD family member 3 (SMAD-3) mRNA was significantly lower in the menstrual endometrium from women with objectively measured HMB versus controls ($p<0.05$) (Figure 92-94). Hairy and enhancer of split-related protein 1 (HEY-1) mRNA did not show statistically significant changes between the two groups in the menstrual phase ($p=0.91$) (Figure 95). Actin-gamma 2 (ACTG2) and isocitrate dehydrogenase 1 (IDH1) mRNA expression was found to be significantly elevated in menstrual endometrium from women with HMB versus normal controls ($p<0.05$) (Figure 96 and 97). Paroxysmal nonkinesiogenic dyskinesia (PNKD) mRNA in menstrual phase endometrium was not significantly different between the two groups (Figure 98).

On examination of these factors in endometrium at all stages of the menstrual cycle from women with HMB/NMB, there was confirmation of significantly lower mRNA of ESM-1 and CXCR4 in women with HMB (Two-way ANOVA) (Figure 92 and 93). SMAD-3 mRNA was not found to be significantly different between women with HMB versus NMB at any stage of the cycle using these analysis parameters. However, there was a non-significant decrease in SMAD-3 expression in women with HMB during the menstrual and proliferative phases (Figure 94). ACTG2 and IDH1 mRNA was not found to be significantly different between women with HMB and NMB when corrected for multiple comparisons using a two-way ANOVA. Interestingly, there were significantly elevated levels of ACTG2 and IDH1 mRNA expression in women with HMB versus normal controls during the early secretory phase (Figures 96 and 97). The impact of the stage of cycle was analysed as a

secondary variable and p-values for the change in mRNA across the cycle for each of the genes examined can be found in Table 19.

Oestrogen receptor 1 (ESR1) was identified in the top scoring networks from GeneGo analysis (Figure 89). ESR1 was mapped on a canonical pathway (green line) and eight genes known to be regulated by ESR1, including SMAD3 and IDH1, were differentially regulated between women with HMB and NMB. Hence, the expression of ESR1 mRNA was also examined by Q-RT-PCR in the endometrium of women with HMB and NMB. There were no significant differences in ESR1 mRNA between women with HMB and normal controls at any stage of the menstrual cycle (Figure 99). However, there was a non-significant increase in ESR1 mRNA during the menstrual phase in women with HMB compared to those with NMB.

As CXCR4 mRNA was confirmed to be significantly increased in menstrual endometrium from women with HMB versus normal controls, the expression of this receptor's ligand, CXCL12 was also examined. There was no significant difference in CXCL12 mRNA between the two groups at any stage of the menstrual cycle (Figure 100).

6.3.7 HIF mRNA in women with HMB

Hypoxia inducible factor (HIF) mRNA was assessed in the endometrium from women with objectively measured HMB and NMB by Q-RT-PCR. HIF-1α mRNA was not significantly different between women with HMB and normal controls at any stage of the menstrual cycle (Figure 101). There was significant variation in HIF-1α mRNA at different phases of the cycle ($p<0.001$). Lowest expression occurred in proliferative phase biopsies with maximal expression during the menstrual phase.

Endometrial HIF-1β mRNA also showed no significant differences between women with HMB and NMB but significant variation between stages of the menstrual cycle ($p<0.0001$) (Figure 102). Again, lowest mRNA levels were observed in proliferative

phase endometrium. Maximal expression occurred in secretory phase biopsies and low expression of HIF-1β mRNA was observed during menstruation.

Table 19. P-values for change in gene expression across the cycle analysed as a secondary variable on two-way ANOVA.

Gene	p-value
ESM-1	0.0036
CXCR4	<0.0001
SMAD3	0.0270
HEY-1	0.4444
ACTG2	0.0346
IDH1	<0.0001
PNKD	<0.0001
ESR-1	<0.0001
CXCL12	0.0099
HIF-1α	0.0005
HIF-1β	<0.0001
VEGF	<0.0001
KDR	0.0009

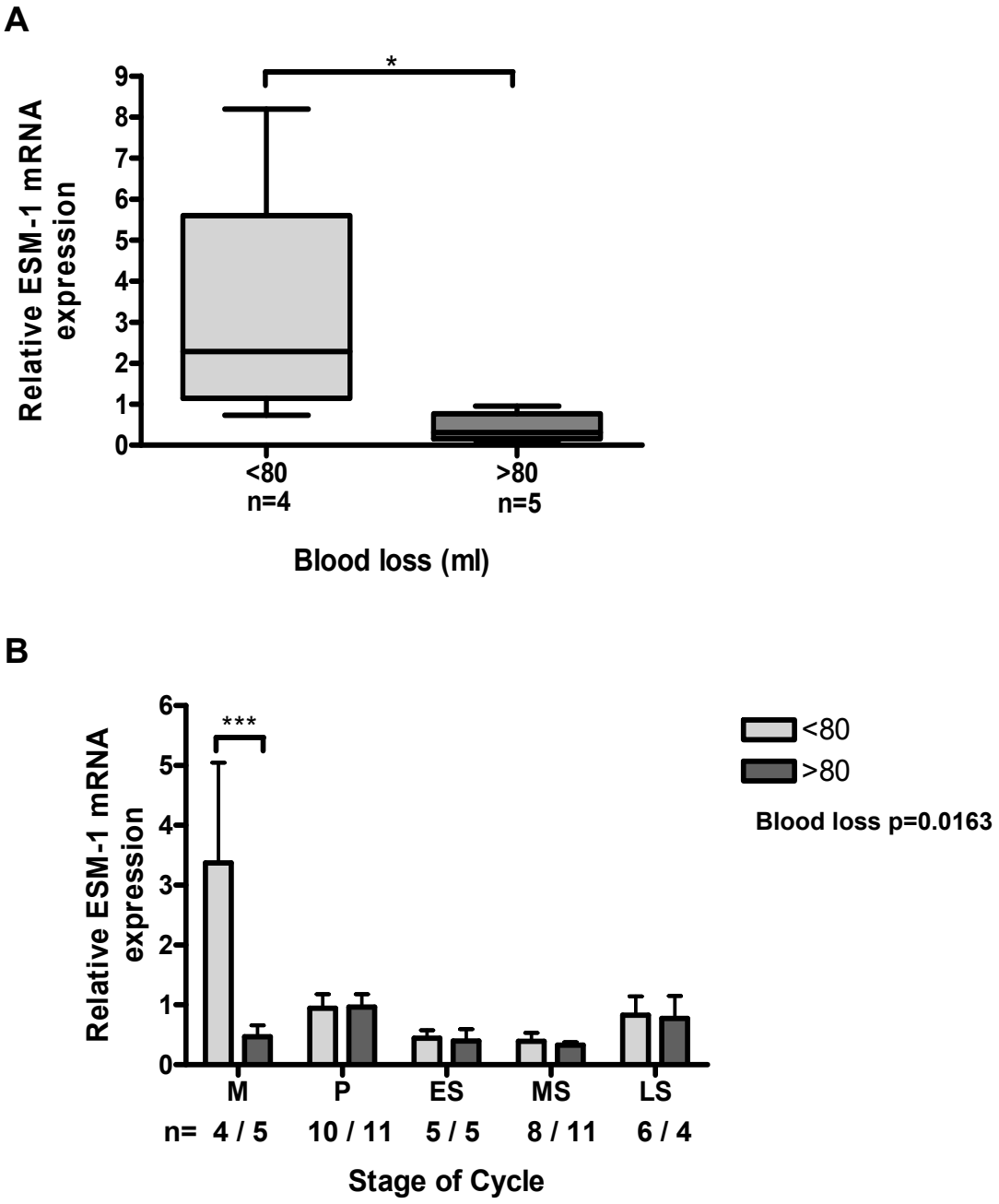


Figure 92. **ESM-1 mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss**. (A) ESM-1 mRNA in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) ESM-1 mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. *p<0.05, ***p<0.001.

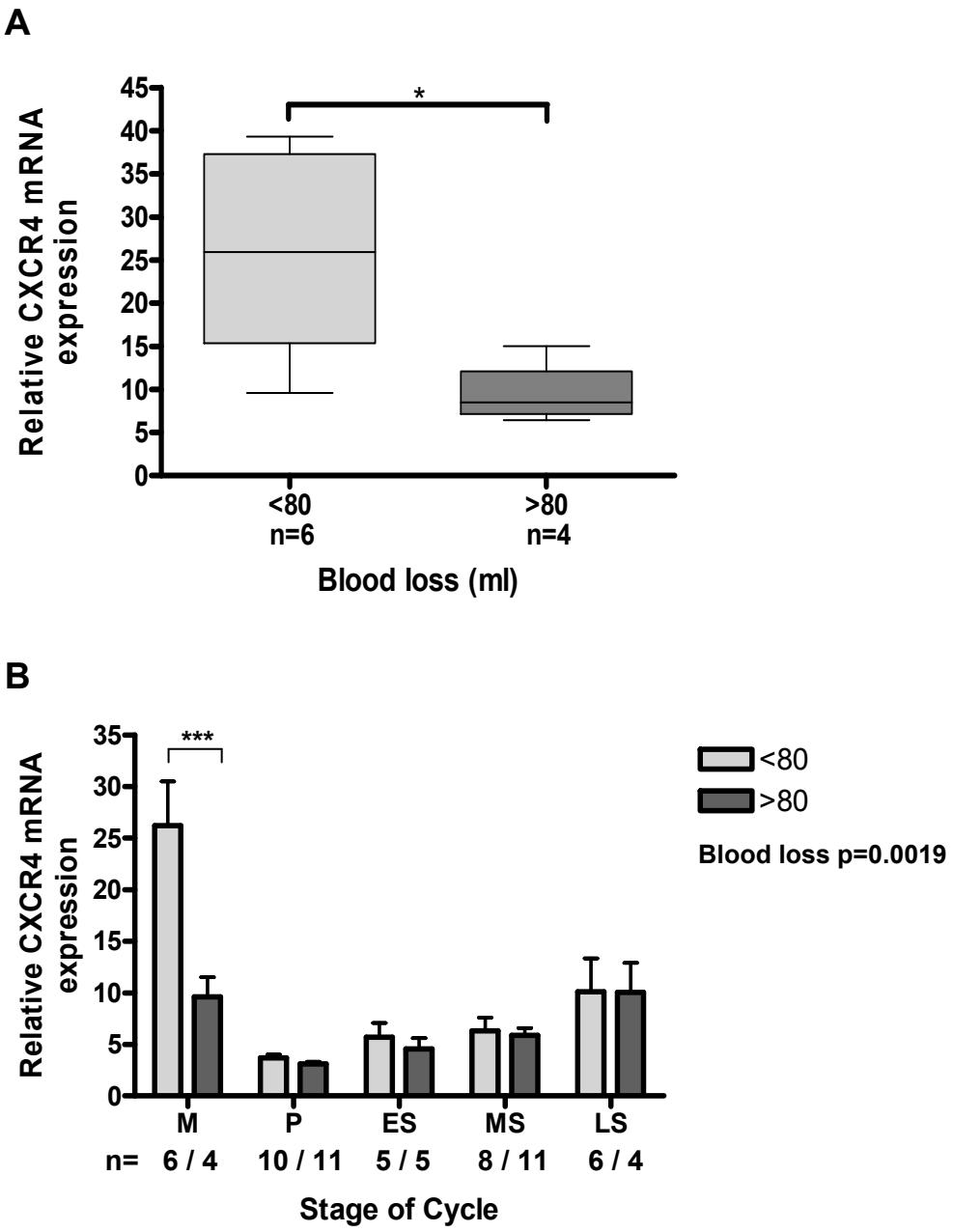


Figure 93. **CXCR4 mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss.** (A) CXCR4 mRNA in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) CXCR4 mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. *p<0.05, ***p<0.001.

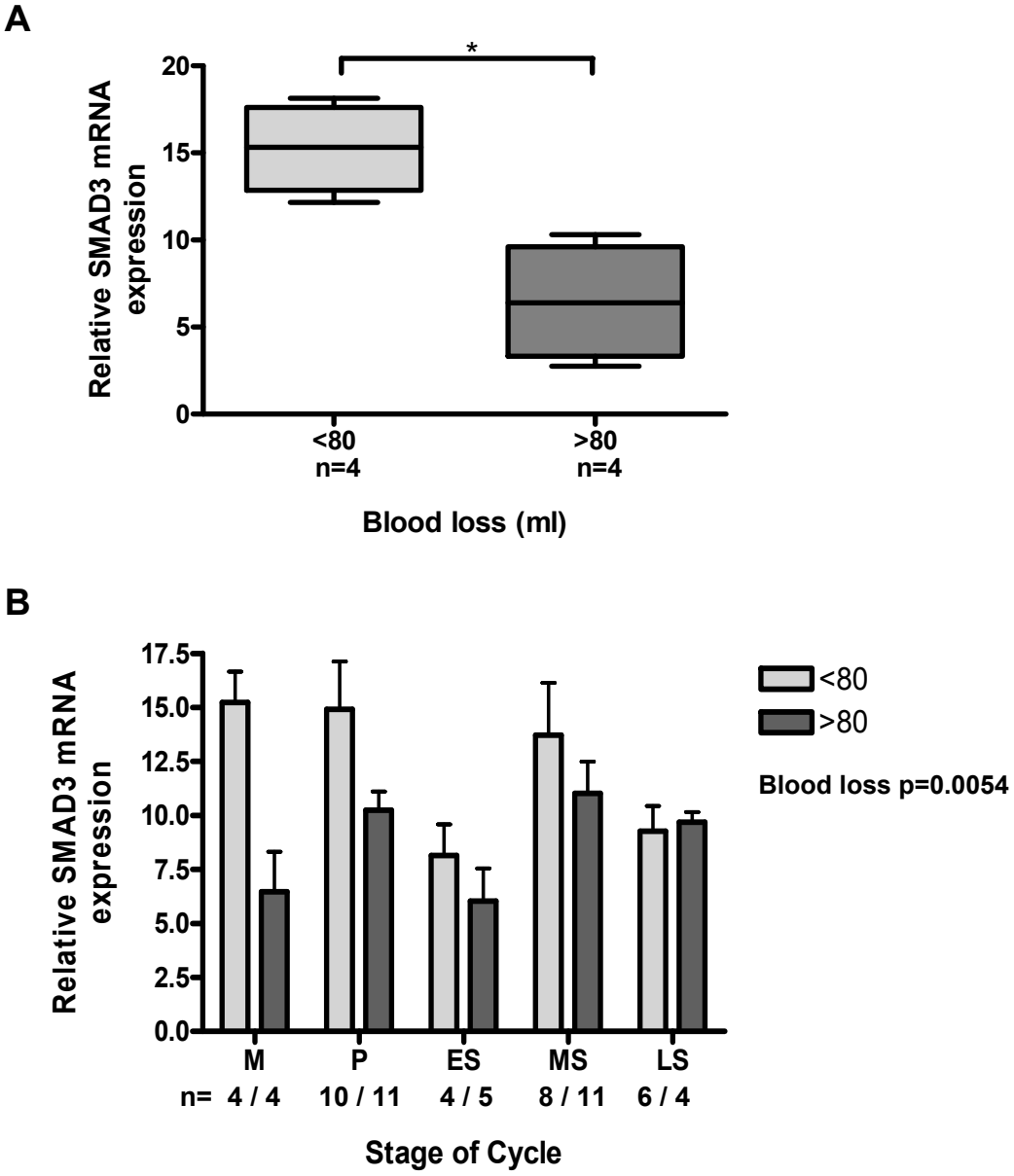


Figure 94. **SMAD3 mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss.** (A) SMAD3 mRNA expression in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) SMAD3 mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. *$p<0.05$.

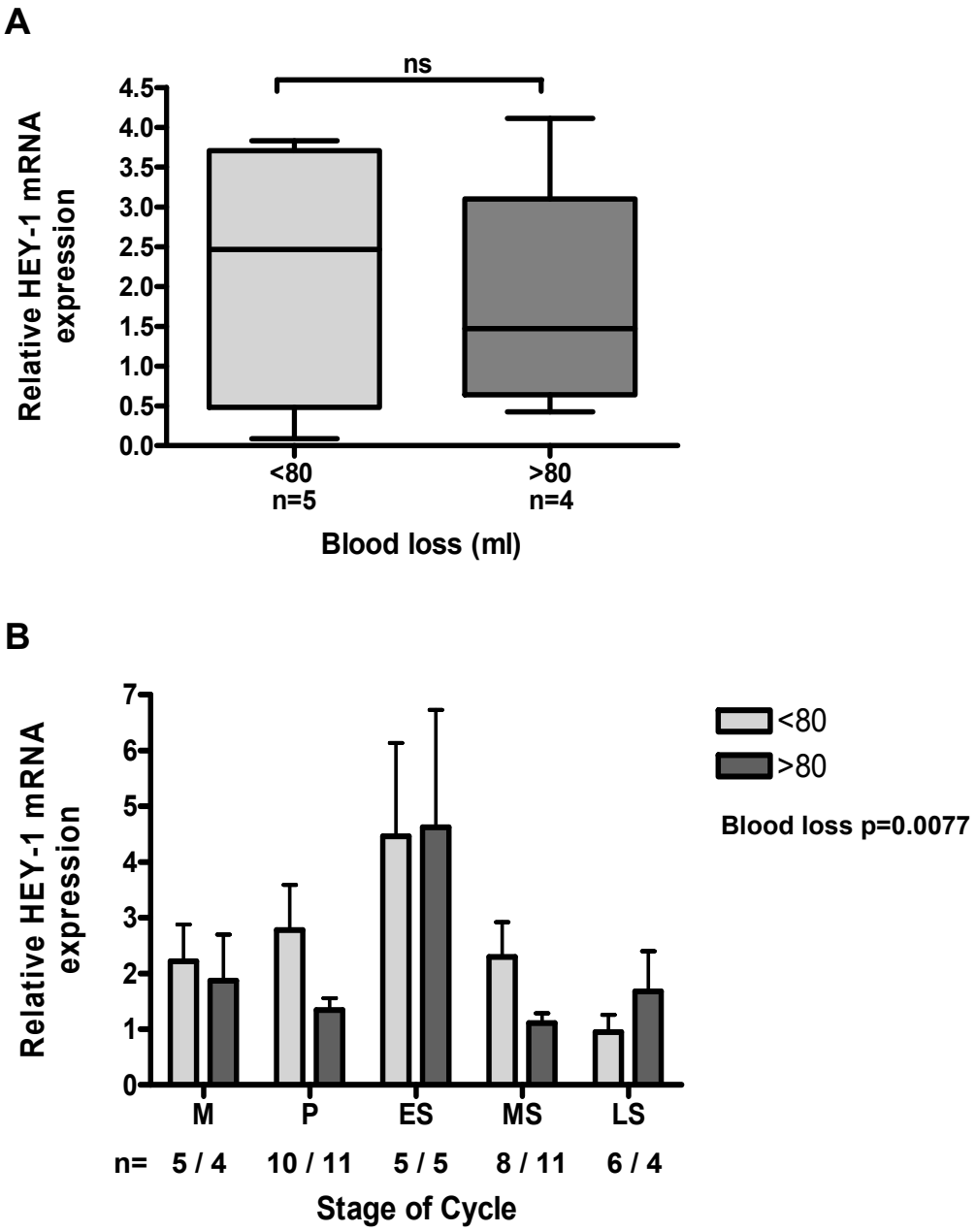


Figure 95. **HEY-1 mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss.** (A) HEY-1 mRNA in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) HEY-1 mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. ns: non-secretory.

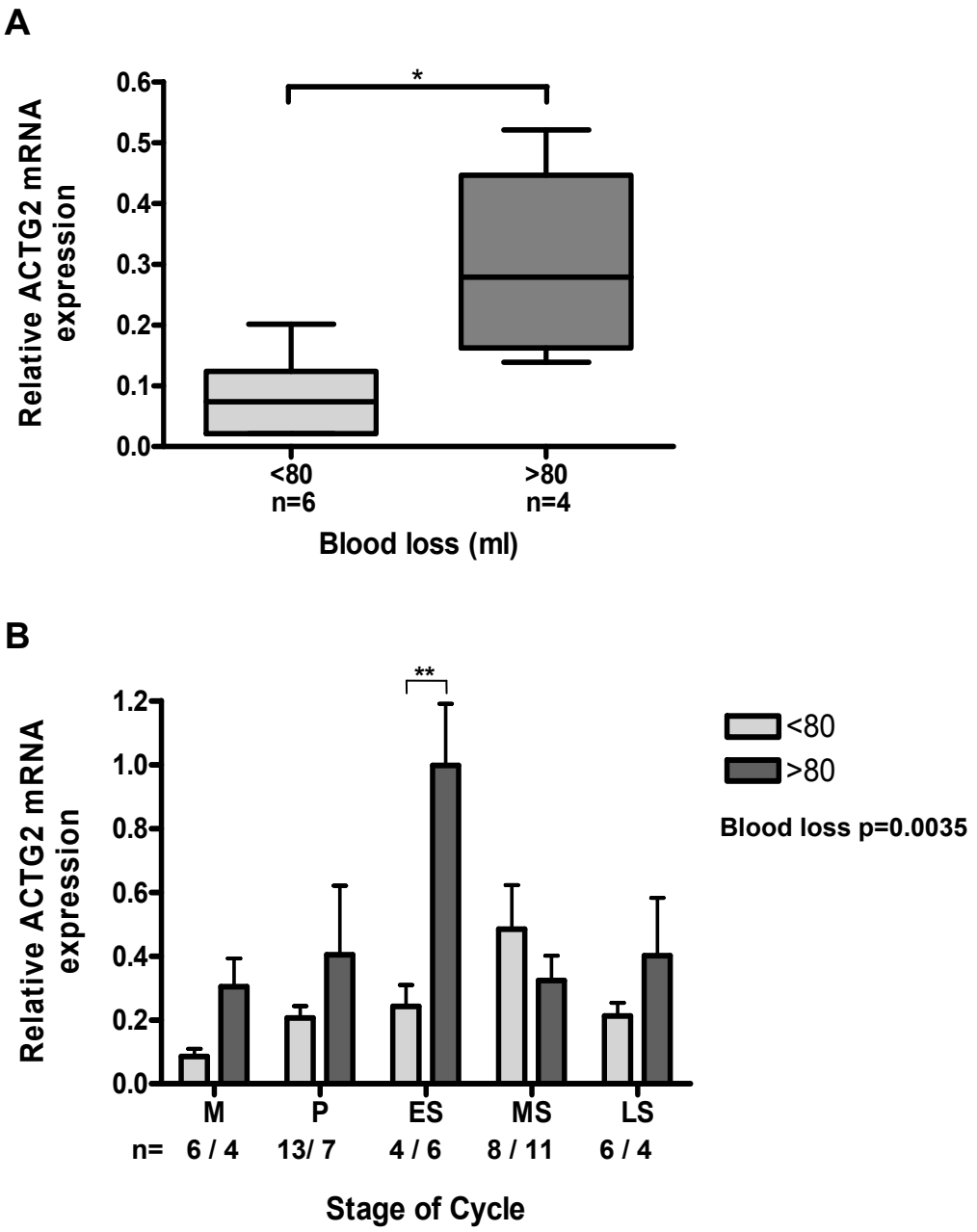


Figure 96. **ACTG2 mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss.** (A) ACTG2 mRNA in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) ACTG2 mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. *p<0.05, **p<0.01.

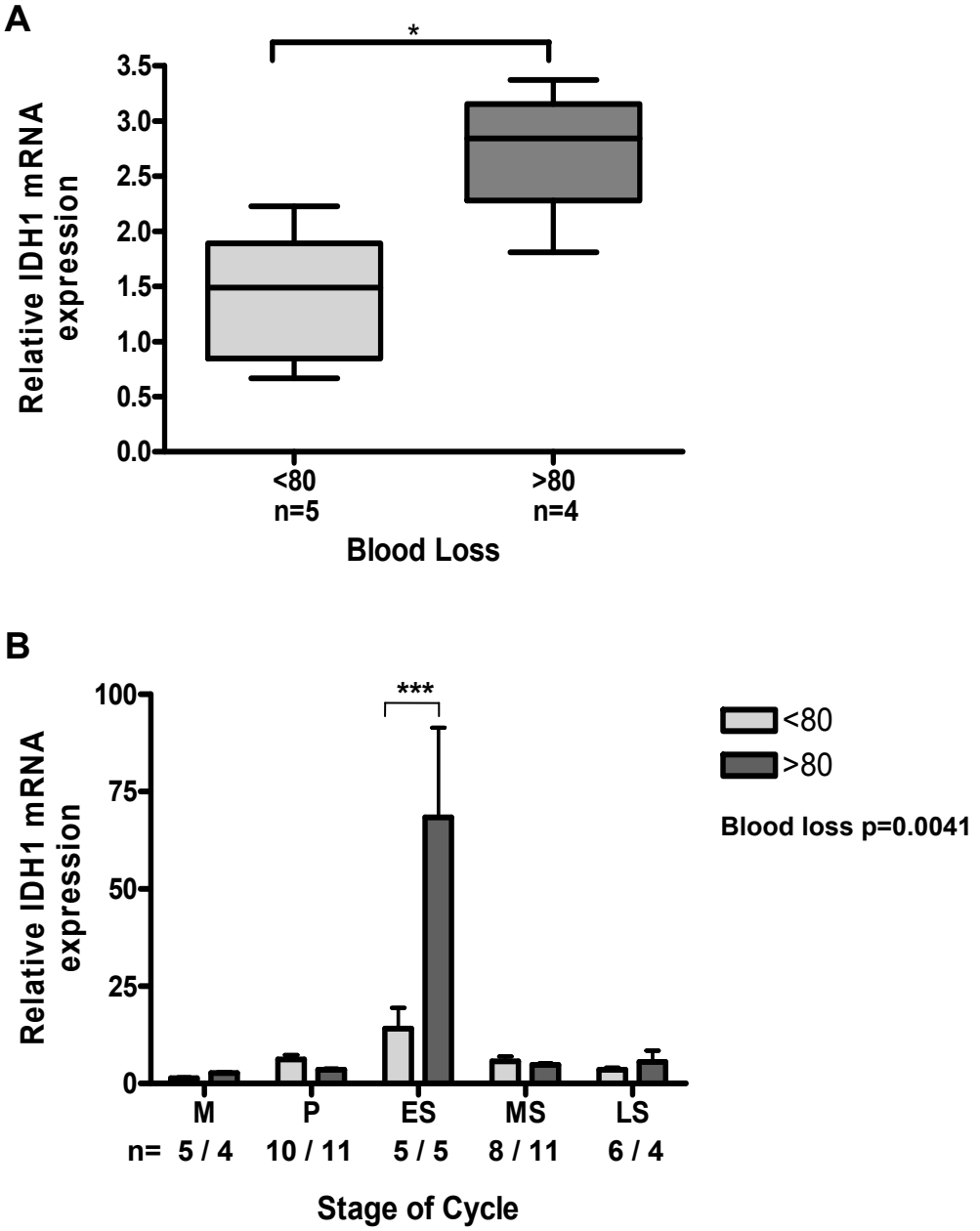


Figure 97. **IDH-1 mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss.** (A) IDH-1 mRNA in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) IDH-1 mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. *p<0.05, ***p<0.001.

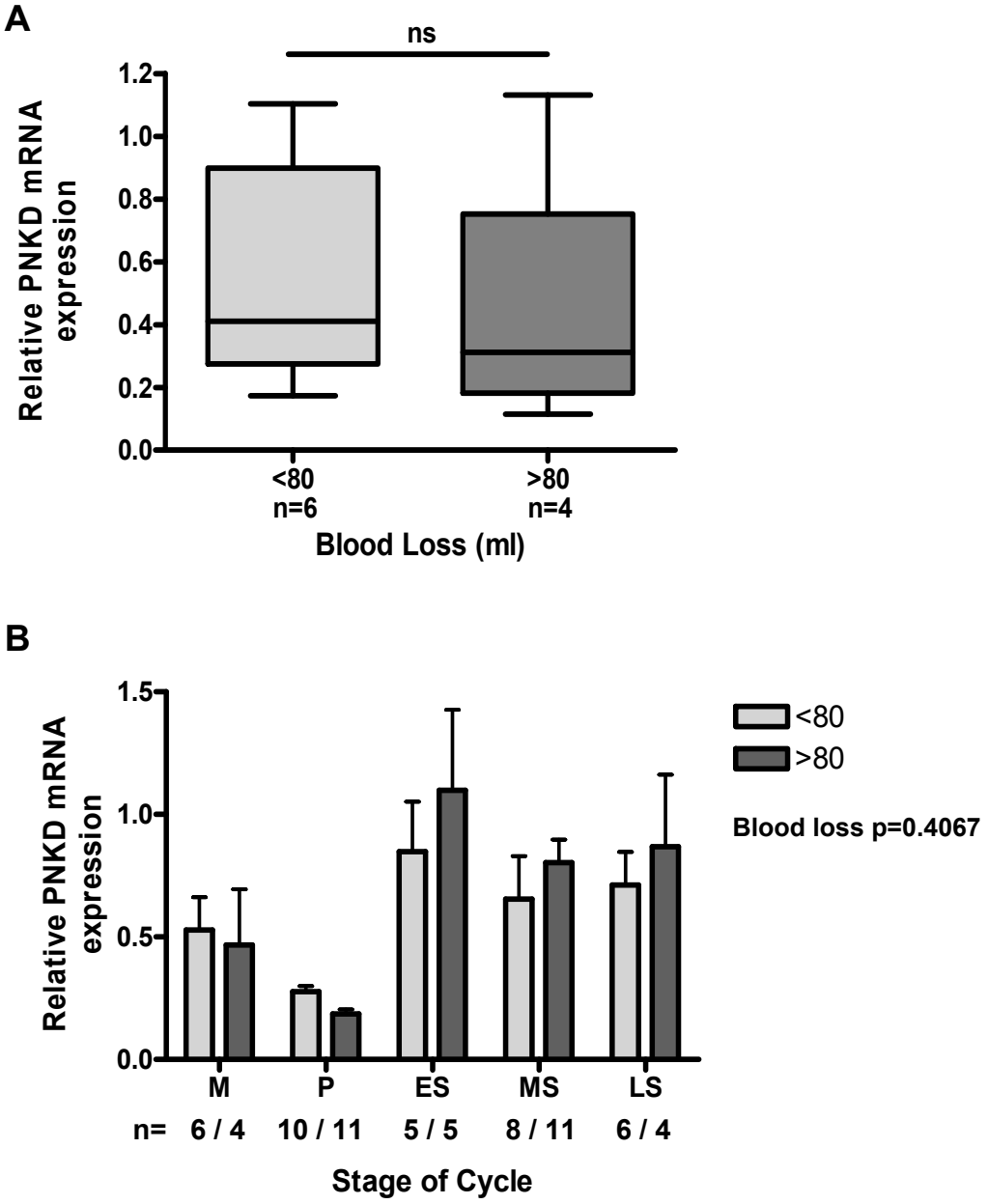


Figure 98. **PNKD mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss.** (A) PNKD mRNA in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) PNKD mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. ns: non-secretory.

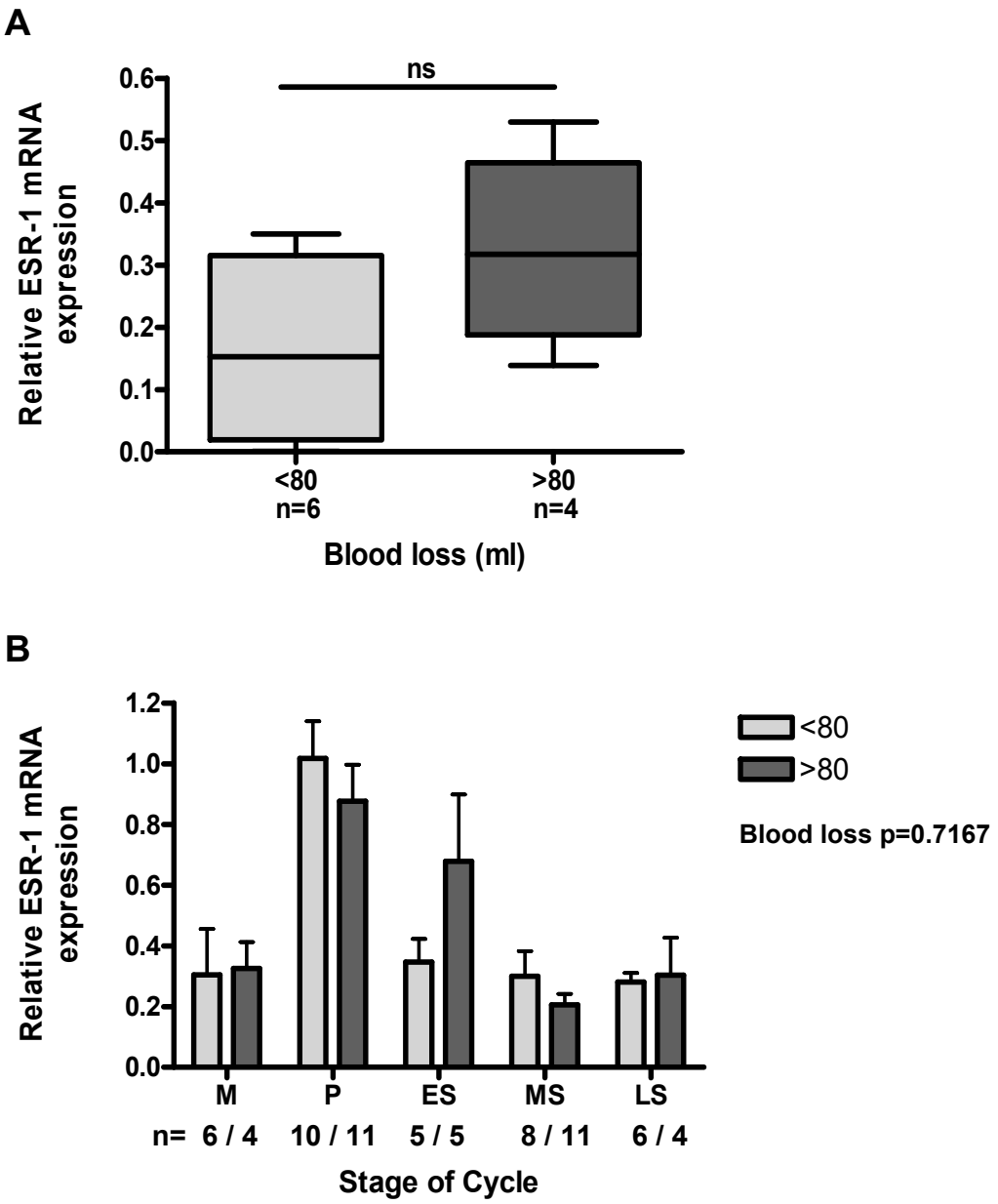


Figure 99. **ESR-1 mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss.** (A) ESR-1 mRNA in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) ESR-1 mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. ns: non-secretory.

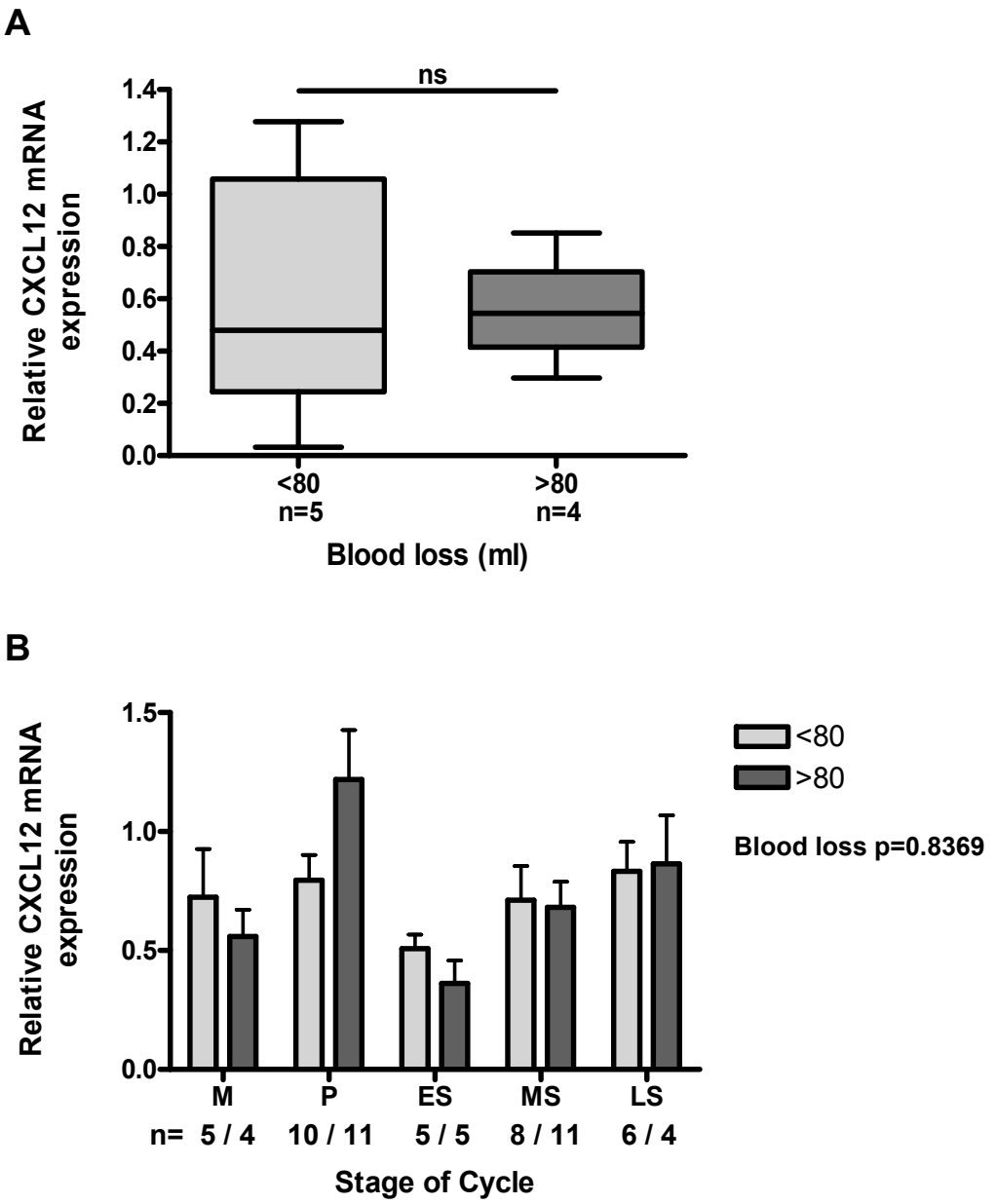


Figure 100. **CXCL12 mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss.** (A) CXCL12 mRNA expression in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) CXCL12 mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. ns: non-significant.

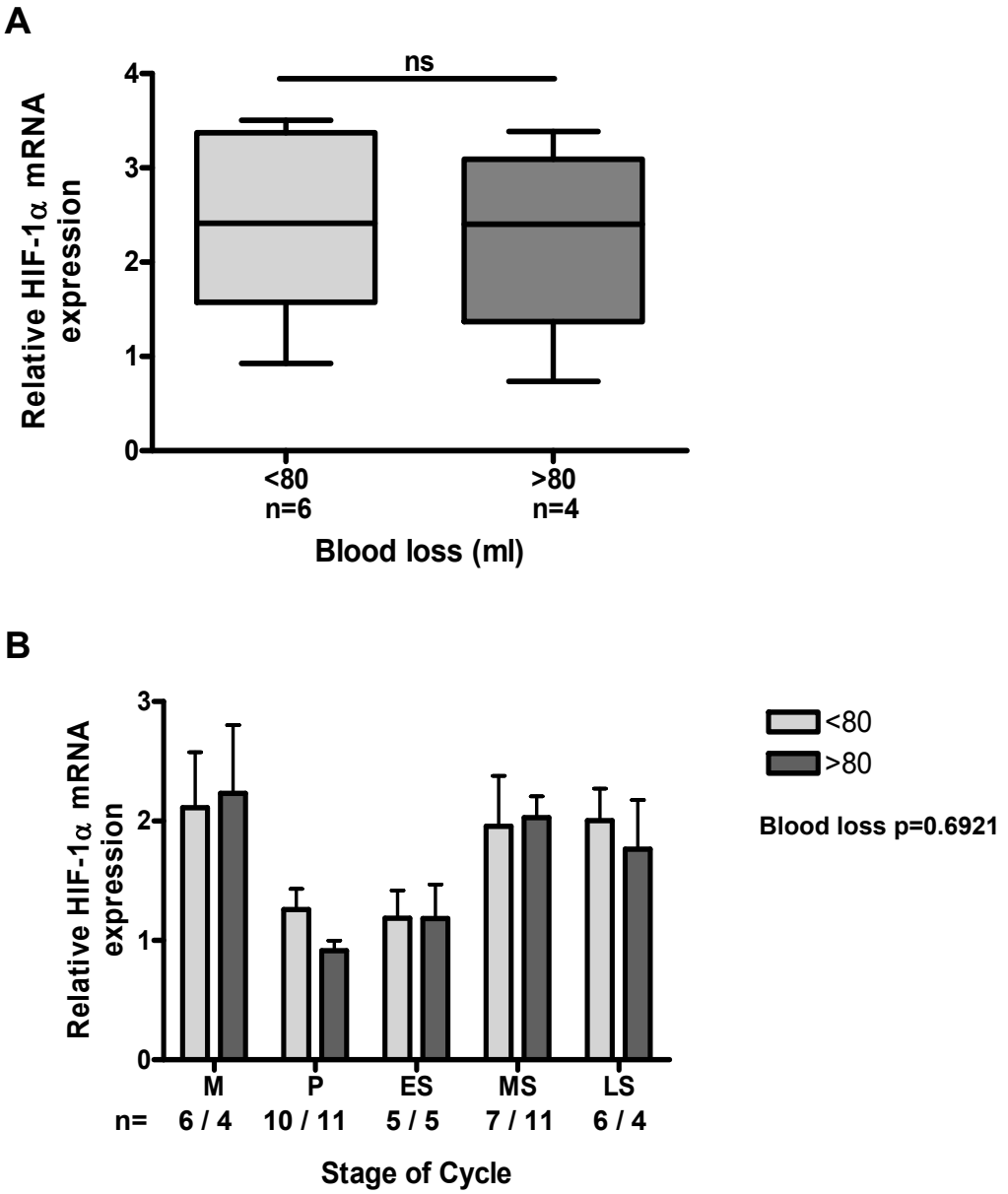


Figure 101. **HIF-1α mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss.** (A) HIF-1α mRNA in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) HIF-1α mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. ns: non-significant.

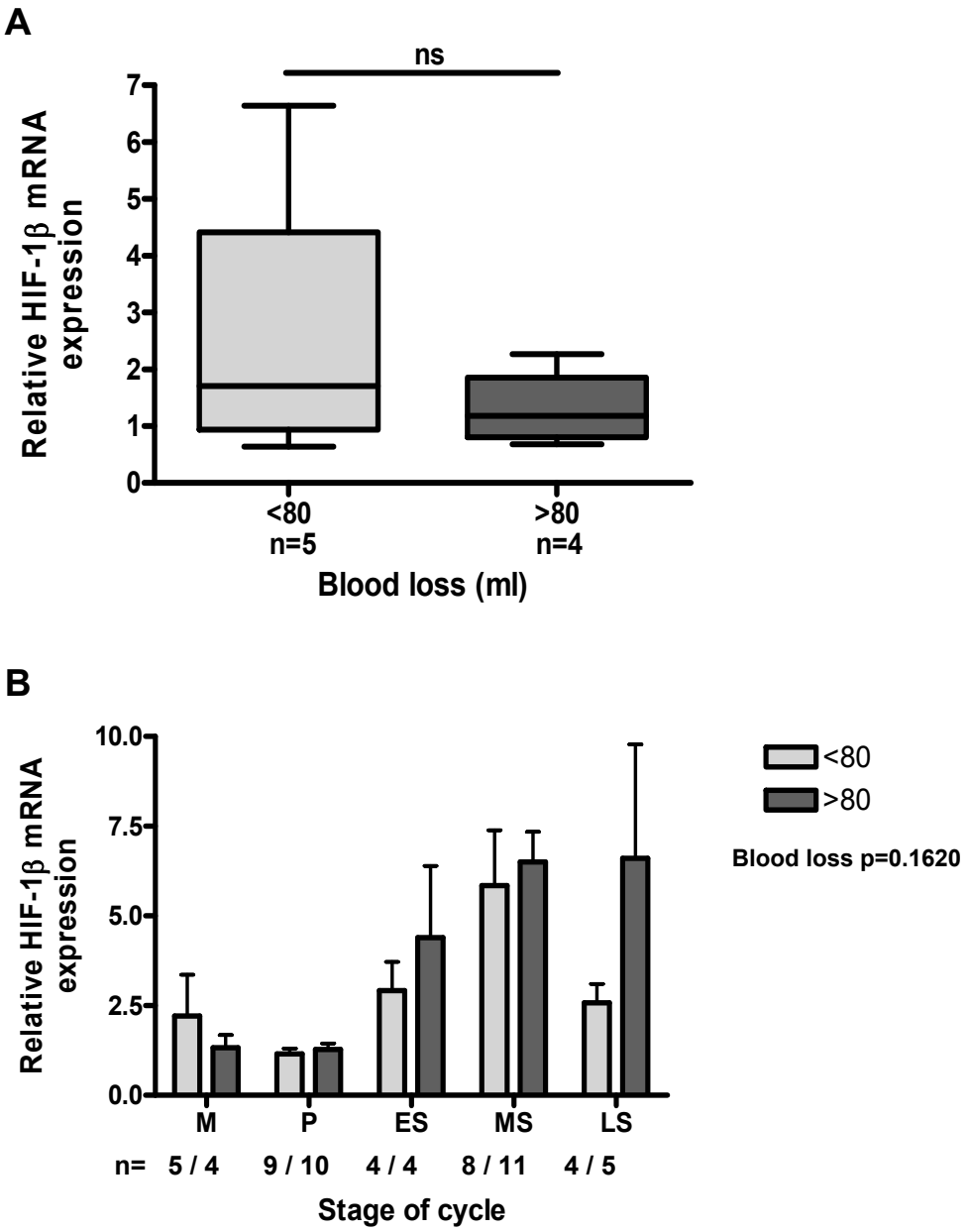


Figure 102. **HIF-1β mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss**. (A) HIF-1β mRNA in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) HIF-1β mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. ns: non-significant.

6.3.8 HIF protein levels in women with HMB

HIF-1α protein levels were assessed in menstrual endometrial biopsies from women with HMB and NMB. Nuclear protein was extracted from eight menstrual biopsies (NMB n=4, HMB n=4) and HIF-1α protein identified by Western blot analysis (Figure 103A). Densitometric quantification revealed significantly lower levels of HIF-1α protein in women with HMB versus normal controls ($p<0.05$) (Figure 103B).

HIF-1β protein levels were assessed by immunohistochemistry in endometrial tissue from across the menstrual cycle. HIF-1β was immunolocalised to the nucleus of endometrial surface epithelial (SE), glandular epithelial (GE) and stromal cells (St) (Figure 107). As evidenced by Figure 104 and 105, HIF-1β staining was maximal during the proliferative phase of the cycle. There was significantly reduced staining of GE, SE and St cells in women with HMB during the proliferative phase when compared to normal controls (Figure 105A). There were no significant differences between these two blood loss groups at any other stage of the cycle (Figure 105B,C).

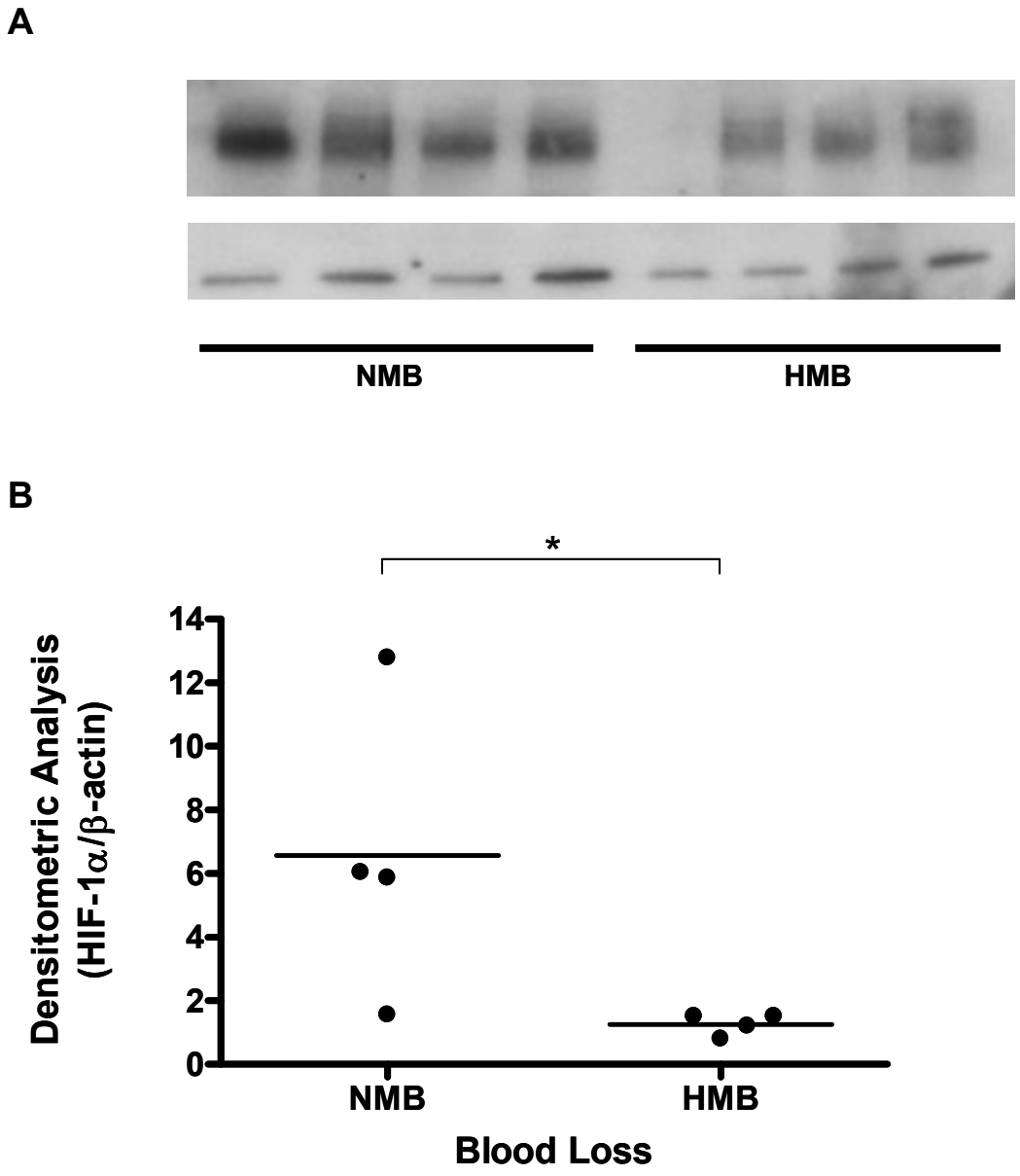


Figure 103. **HIF-1α protein in menstrual endometrium from women with objectively measured menstrual blood loss**. (A) Western blot for HIF-1α and β-actin in menstrual phase endometrium from women with heavy menstrual bleeding (HMB: blood loss >80ml) and normal controls (NMB: blood loss <80ml). (B) Densitometric analysis of HIF-1α protein normalised against β-actin. *$p<0.05$.

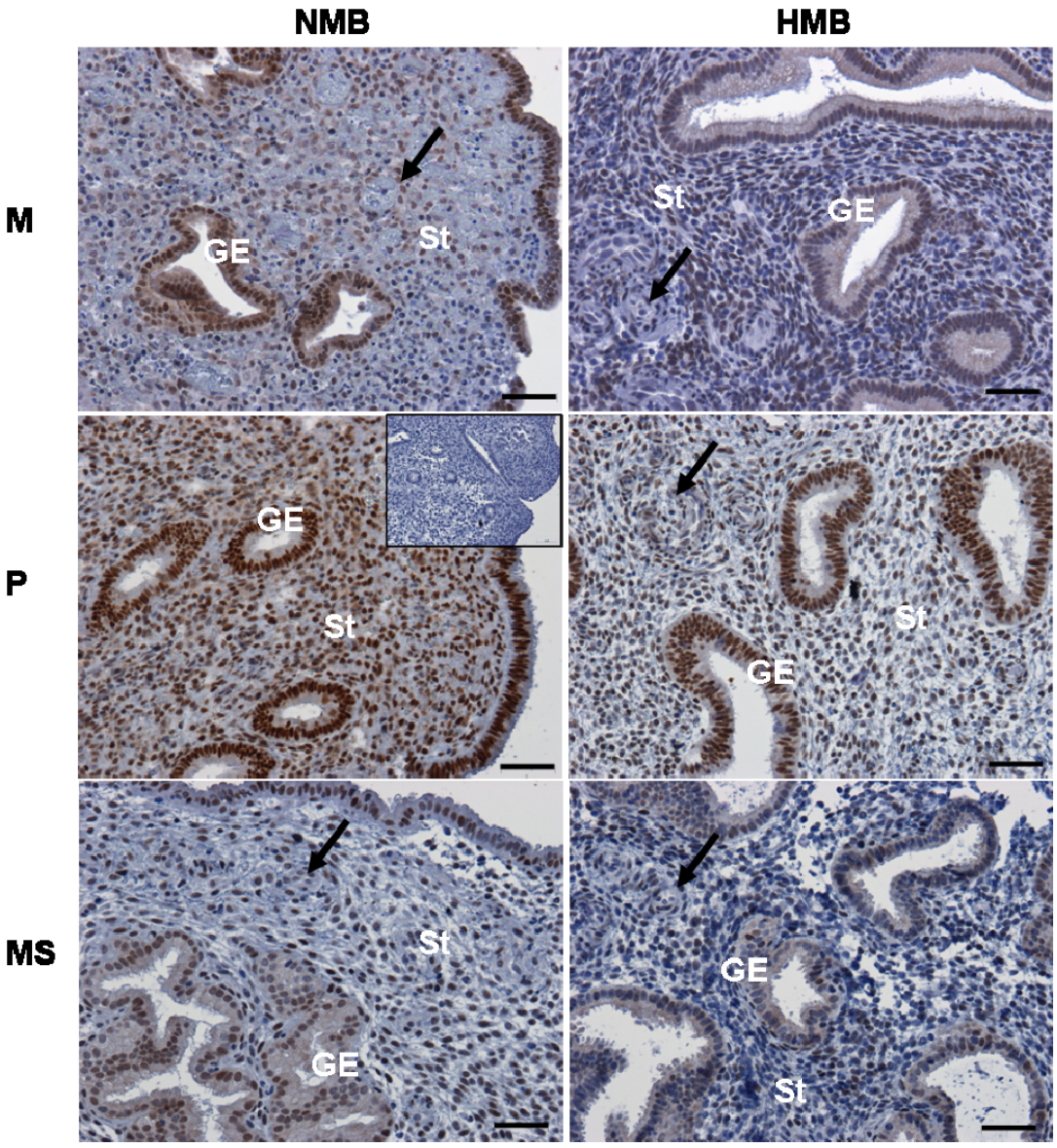


Figure 104. **Immunohistochemical staining for HIF-1β in the human endometrium.** SE: surface epithelial cells, GE: glandular epithelial cells, St: stromal cell compartment, arrow: perivascular cells, scale bar: 50μm. NMB: normal menstrual bleeding (<80ml), HMB: heavy menstrual bleeding (>80ml). M: menstrual, P: proliferative, MS: mid secretory.

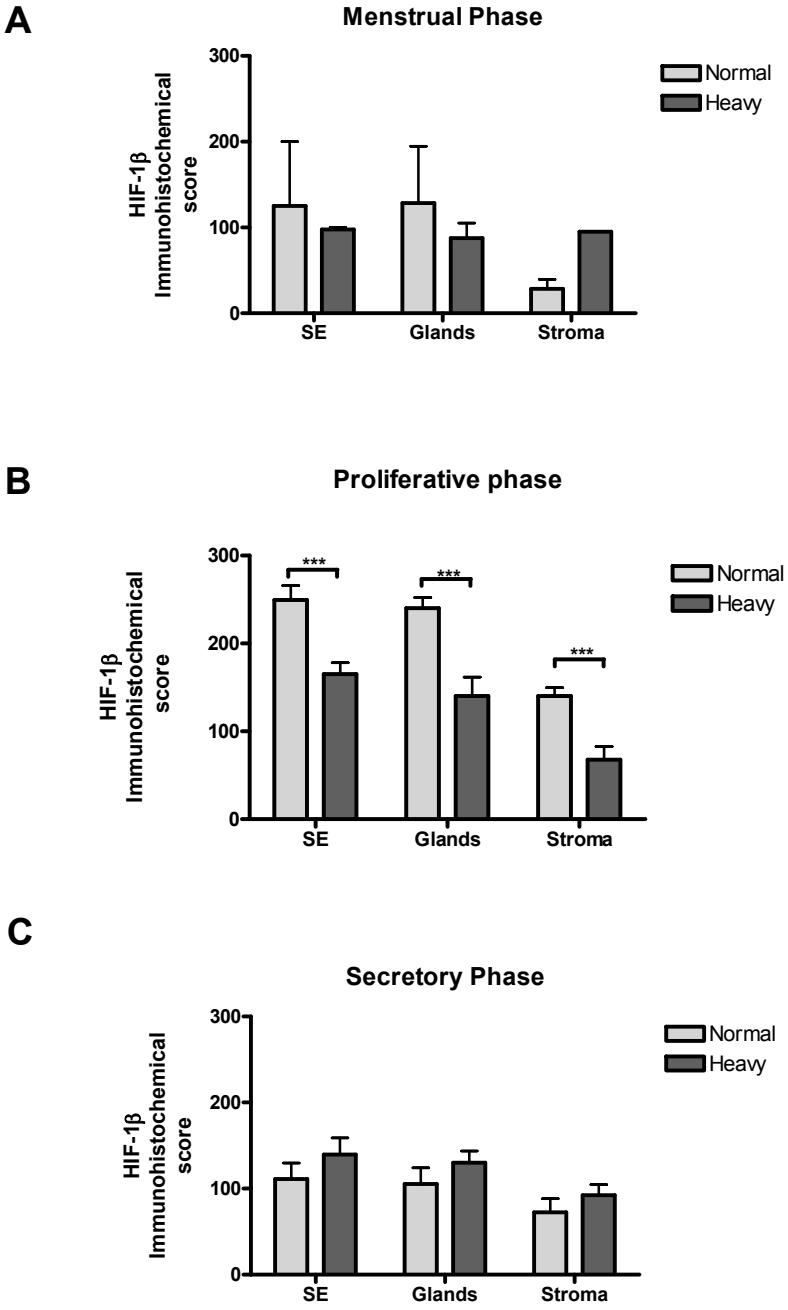


Figure 105. **Semi-quantitative histoscoring of HIF-1β staining in endometrium from women with objectively measured menstrual blood loss.** HIF-1β staining scores in (A) menstrual endometrium (B) proliferative endometrium and (C) secretory phase endometrium from women with HMB (blood loss >80ml) and normal controls (blood loss <80ml). SE: surface epithelial cells, ***p<0.001.

6.3.9 VEGF levels in women with HMB

As a known downstream target of HIF-1, vascular endothelial growth factor (VEGF) levels were examined in the endometrium of women with HMB and NMB. VEGF mRNA levels were significantly decreased in the menstrual endometrium of women with HMB versus normal blood loss controls ($p<0.05$) (Figure 106A). When examining VEGF mRNA across the menstrual cycle, there were low levels of expression during the proliferative and secretory phases. There was only significant up-regulation of VEGF mRNA at menstruation in women with NMB ($p<0.001$) (Figure 106B).

VEGF protein levels were examined using immunohistochemistry. VEGF was immunolocalised to the surface epithelium, glandular epithelium, stromal and endothelial cells (Figure 107). VEGF immunohistochemical staining was less marked in the menstrual endometrium from women with HMB versus NMB (Figure 107). Menstrual endometrium from women with HMB (n=2) and NMB (n=2) were cultured *in vitro* for 24h. Although numbers were small, supernatants subjected to ELISA analysis revealed women with HMB had lower levels of secreted VEGF protein than controls (Figure 108).

Expression levels of one of the VEGF receptors, kinase insert domain receptor (KDR), were also examined in endometrium from women with HMB/NMB. Although there were significant changes in KDR mRNA across the cycle, with maximal expression in menstrual phase biopsies, there was no significant difference in expression between women with HMB and normal controls (Figure 109).

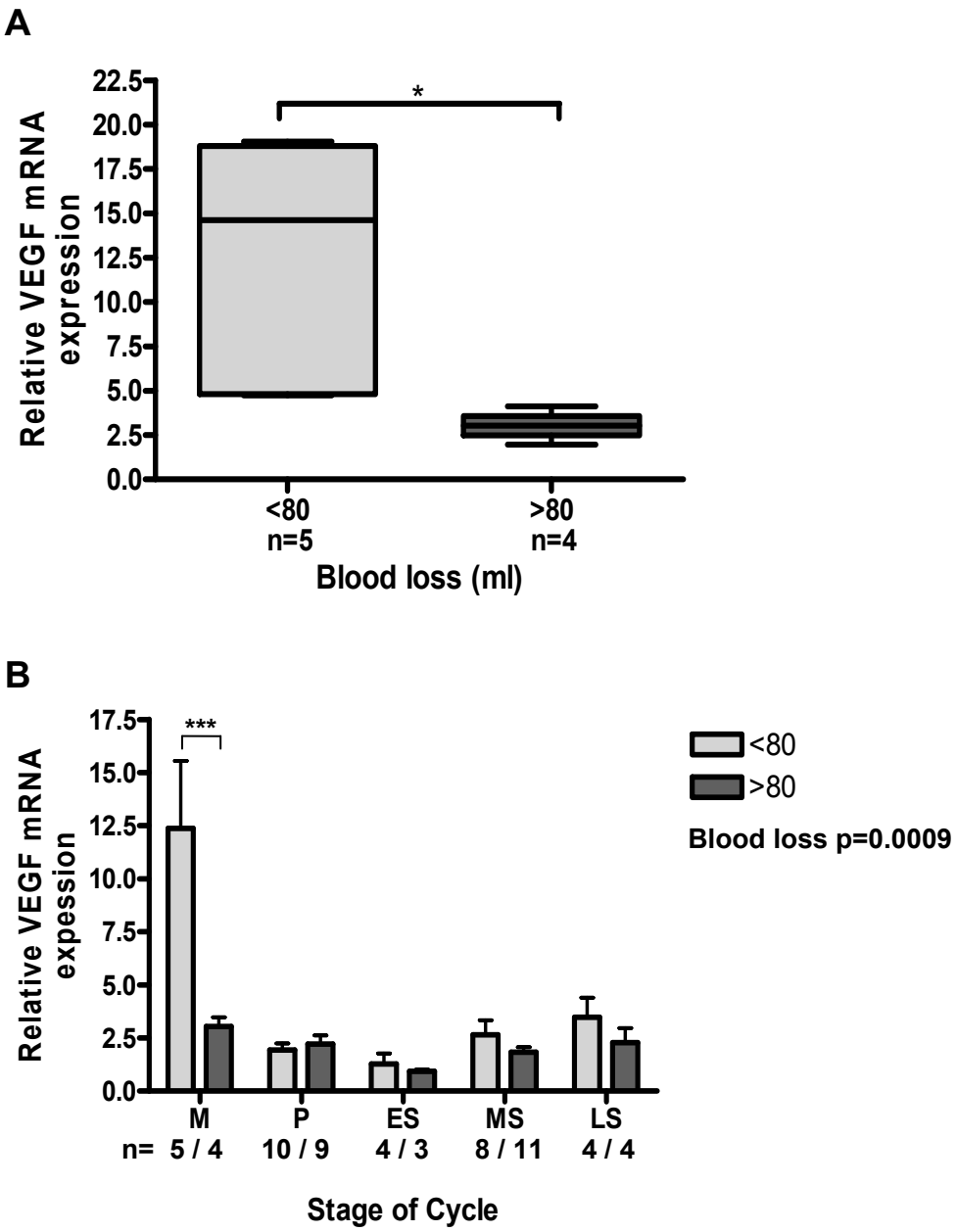


Figure 106. **VEGF mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss.** (A) VEGF mRNA in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) VEGF mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. *p<0.05, ***p<0.001.

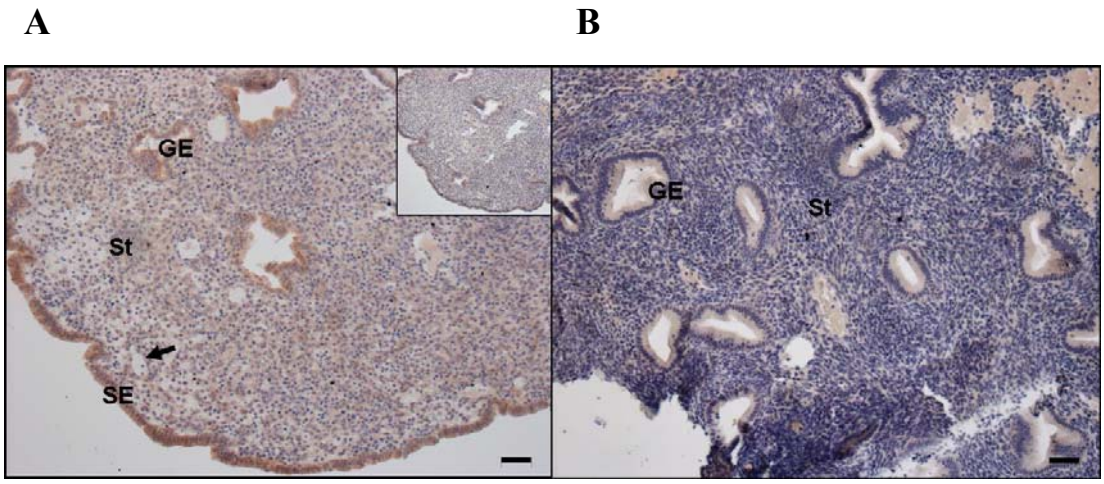


Figure 107. **VEGF immunolocalisation in the menstrual endometrium of women with heavy and normal menstrual blood loss**. (A) menstrual endometrium from a woman with normal menstrual bleeding (<80ml). (B) menstrual endometrium from a woman with heavy menstrual bleeding (>80ml). GE: glandular epithelial cells, SE: surface epithelial cells, St: stromal cell compartment, arrow: perivascular cells, scale bar = 50μM. Insert = negative control.

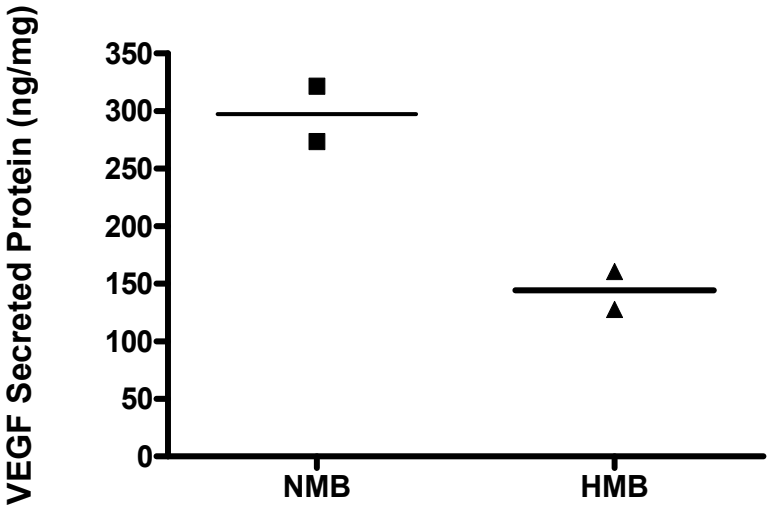


Figure 108. VEGF secreted protein levels in culture supernatants of endometrial tissue explants from women with heavy (HMB) and normal (NMB) menstrual bleeding incubated for 24h *in vitro*.

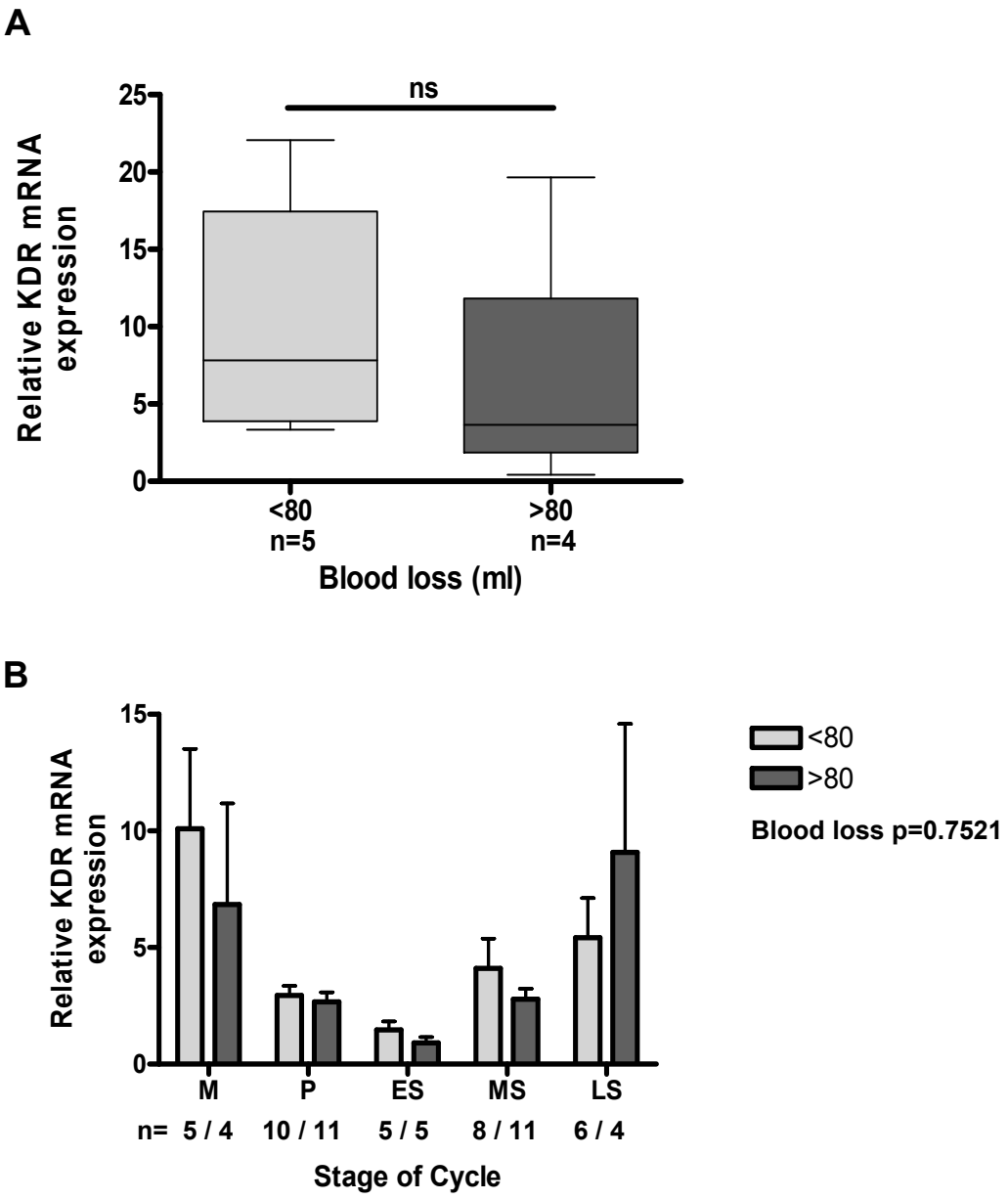


Figure 109. **KDR mRNA in the endometrium of women with objectively measured normal and heavy menstrual blood loss.** (A) KDR mRNA in menstrual endometrium from women with normal (<80ml) and heavy (>80ml) menstrual blood loss. (B) KDR mRNA in endometrium from across the menstrual cycle in women with heavy and normal loss subjected to two-way ANOVA. M: menstrual, P: proliferative, ES: early secretory, MS: mid secretory, LS: late secretory. ns: non-secretory.

6.3.10 Angiogenic potential of endometrial HIF-1α

An *in vitro* tube formation assay was utilised to assess the angiogenic potential of endometrial HIF-1α. Due to the limitations of human tissue samples, an endometrial epithelial cell line was used. HIF-1α was silenced in these cells using two separate ShRNA constructs. Conditioned media from untransfected cells and cells transfected with (i) HIF-1α/ShRNA1470, (ii) HIF-1α/ShRNA2192 or (iii) a scrambled sequence HIF-1α/ShRNASCR were used to treat HUVECs. There was a significant increase in HUVEC branching at 8h with culture supernatant from untransfected endometrial epithelial cells cultured in hypoxic versus normoxic conditions (Figure 110). Culture supernatant from endometrial cells transfected with a scrambled ShRNA sequence before incubation in hypoxia also significantly induced HUVEC branching ($p<0.05$). This increased HUVEC angiogenic response was abolished when HIF-1α was silenced in endometrial cells before culture in hypoxic conditions ($p<0.05$). To assess the contribution of VEGF, 300ng/ml of VEGF protein was added to the culture supernatant from HIF-1α silenced cells before incubation with HUVEC cells. This concentration of VEGF is equivalent to the levels secreted by menstrual endometrial biopsies (per mg) from women with NMB (Figure 108). Replacement of VEGF partially restored the tube formation ability of hypoxic endometrial cell supernatant, but branch counts were not significantly higher than those from HIF-1α silenced cells.

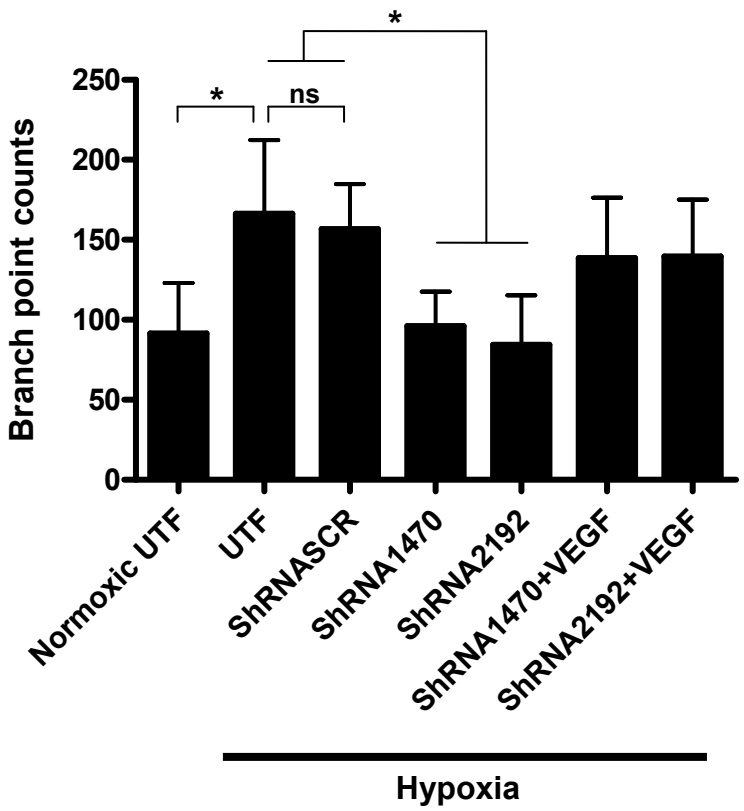


Figure 110. **Angiogenic potential of endometrial HIF-1α production.** Culture supernatant from endometrial cells incubated in hypoxia demonstrated increased endothelial branching versus normoxic supernatants. This response was lost when endometrial cell HIF-1α was silenced prior to hypoxic incubation. Replacement of VEGF, at levels equivalent to those in women with normal bleeding, partially restored the angiogenic response. UTF: untransfected cells, Hypoxia: 0.5% O_2, Normoxia: 21% O_2, SCR: scrambled ShRNA sequence, 1470/2192: ShRNA against HIF-1α, VEGF: vascular endothelial growth factor.

6.4 Discussion

This chapter contributes to existing knowledge of the pathogenesis of HMB. Firstly, it reveals the novel findings of an unbiased whole genome array, detailing significant differences in the menstrual endometrium of women with HMB and NMB. Secondly, results described herein reveal that HIF-1α protein levels are significantly decreased in the endometrium of women with HMB when compared to normal controls. In addition, expression of the HIF-1 target genes VEGF and CXCR4 follow a similar pattern, with significantly lower levels in women with objectively measured HMB versus women with NMB. Furthermore, the functional consequences of defective HIF-1α protein levels were examined using short-hairpin RNA. These studies revealed a significant decrease in endometrial angiogenic potential when HIF-1α production was inhibited.

The gene expression profiling study of menstrual endometrium from women with NMB and HMB described herein confirms that endometrial tissue is amenable to this method of study. Notwithstanding the heterogeneity of the tissue and the considerable biological differences that exist between individual women, significant differences in endometrial gene expression were detected and validated between the two groups of women. These findings concur with other endometrial microarray studies, which have also demonstrated endometrial segregation into clusters for stage of cycle and for the presence or absence of endometriosis (Ponnampalam et al., 2004, Critchley et al., 2006c, Burney et al., 2007).

Unbiased functional analysis of the differently expressed genes in the menstrual endometrium of women with NMB and HMB revealed novel processes that may be aberrant in women with heavy blood loss. *In silico* analysis allowed the formation of network and sub-network objects. These networks are descriptive of a biological function and detail known interactions between genes, reflecting the multi-functional activity of some factors. In this way, differences in gene expression between women with HMB and NMB were grouped, allowing the complex myriad of differentially regulated genes to reflect possible biological dysfunction.

The top ranked GeneGo process network was apoptosis. In addition, a number of significant sub-networks contained GO processes such as “regulation of cell death” and “programmed cell death”. The presence of differentially expressed genes in these networks suggests that apoptosis may be aberrant in the menstrual endometrium of women with HMB. Electron microscopy studies of the normal human endometrium have identified apoptotic bodies in the late secretory and menstrual phases of the cycle, with decreasing levels during the early proliferative phase (Hopwood and Levison, 1976). B cell lymphoma 2 (bcl-2) is an apoptosis regulator protein and is found in the human endometrium (Otsuki et al., 1994). Interestingly, its detection in the glandular epithelial cells was limited to the proliferative and early secretory phases, with decreased detection in the late secretory and menstrual phase. This decreased staining correlated with the appearance of apoptotic cells, suggesting bcl-2 prevents or limits apoptosis in the human endometrium. There is recent evidence that androgens are also involved in suppression of apoptosis during the proliferative phase, to facilitate post-menstrual remodelling of the endometrium (Marshall et al., 2011). It appears that a balance of proliferation and apoptosis is necessary for normal endometrial function throughout the cycle. Considering its key role in endometrial physiology, excessive or prolonged apoptosis in the endometrium at menstruation may have a significant impact on menstrual bleeding. Interestingly, analysis of proliferative endometrium of women with a subjective complaint of HMB revealed significantly higher levels of apoptotic bodies than in endometrium from normal controls (Stewart et al., 1999). The finding of aberrant expression of several factors with a known role in apoptosis in the menstrual endometrium of women with HMB provides a novel field of research.

This chapter details validation of the down regulation of SMAD-3 and the up-regulation of ACTG2 and IDH1 in women with HMB versus normal controls. These three genes appear on GeneGo subnetwork 2 (Figure 89), which details gene interactions involved in regulation of signalling and cellular processes. ESR1 mRNA was not significantly increased in women with HMB in this study. ESR1 negatively regulates SMAD3 (red arrow, Figure 89) (Blanchette et al., 2006) and is known to

have a binding site in the IDH1 promoter region (Gao et al., 2008). Therefore a role in the down regulation of SMAD3 and up-regulation of IDH1 in women with HMB cannot be excluded. Furthermore, ACTG2 appears downstream of SMAD3 on this gene network (Figure 89) and has also been validated as being differentially expressed in women with HMB/NMB. An additional downstream target of SMAD-3 is ESM-1, as detailed in Figure 93. ESM-1 was also validated as being decreased in women with HMB versus controls, consistent with less positive regulation due to decreased expression of SMAD-3.

Subnetwork 24 includes CXCR4; the most significantly down regulated gene on the microarray study of menstrual endometrium from women with HMB versus NMB (Figure 91). This network details gene interactions that are involved in regulation of biological processes and apoptosis. Signalling of CXCL12 through CXCR4 in trophoblast cell cultures has been shown to stimulate anti-apoptotic pathways and increase cell survival (Jaleel et al., 2004, Wu et al., 2004). CXCR4 has recently been identified in the human endometrium, with significantly higher mRNA during the early proliferative phase (Laird et al., 2011). CXCR4 protein was identified by immunohistochemistry in the epithelium and blood vessels, with weaker staining in stromal cells. There were no obvious staining patterns across the cycle but this study did not include endometrium from the menstrual phase. The promoter region of CXCR4 contains the hypoxic response element that binds HIF-1α (Ishikawa et al., 2009, Pan et al., 2006). HIF-1α does not appear in the differentially regulated gene list in women with HMB and NMB, which is unsurprising considering its posttranslational regulation. However, P300, a cofactor for HIF-1α that facilitates HIF-1 transactivation, features on subnetwork 24. This interacts with a number of differentially expressed genes, including CXCR4, SMAD3 and ETS2, a member of a family of transcription factors known to be involved in the up-regulation of hypoxia induced genes (Aprelikova et al., 2006).

Assessment of the protein levels of all of these Q-RT-PCR validated factors is required. In addition, validation of other differentially expressed genes in these GeneGo networks is necessary, using Taqman Q-RT-PCR and protein quantification

methods. Furthermore, delineation of a functional difference in apoptotic activity in the menstrual endometrium of women with HMB and NMB would provide evidence for its involvement in the pathophysiology of HMB.

Functional analysis of the differentially expressed genes using globaltest GO annotations identified "response to hypoxia" as the 7th ranked process (Table 16). There were 72 genes in the differentially expressed list that were functionally related to this process (Figure 87). This finding, coupled with the data presented in chapter 5, suggested that hypoxia-inducible factor may be aberrant in the endometrium of women with HMB.

On examination of mRNA, there were no significant differences in HIF-1α or HIF-1β expression between women with NMB and HMB at any stage of the menstrual cycle. These findings are consistent with the well documented evidence for post-translational regulation of HIF-1α protein (Ivan et al., 2001, Jaakkola et al., 2001). The contribution of transcriptional and translational mechanisms to HIF-1 regulation remains unclear. There are some reports of hypoxia increasing the transcription of HIF-1α (Belaiba et al., 2007) and others that report a significant decrease in HIF-1α mRNA in prolonged hypoxic conditions (Uchida et al., 2004). In contrast, the dominant mechanism of non-hypoxic regulation of HIF-1 appears to be increased transcription or translation by inflammatory mediators or growth hormones (van Uden et al., 2008, Frede et al., 2005, Dery et al., 2005). Therefore, even if there is a marked difference in oxygen levels between the menstrual endometrium of women with NMB and HMB, HIF-1α mRNA may not reflect this.

As the presence of HIF-1α protein appears to be limited to the pre-menstrual and menstrual phases of the cycle (Chapter 5, Figure 63)(Critchley et al., 2006b), levels of this protein were examined in menstrual phase endometrium from women with objectively measured MBL. Densitometric analysis of Western blot data revealed significantly lower levels of HIF-1α protein in the endometrium of women with HMB versus normal controls. This novel finding provides new insight into the role of HIF-1 in endometrial physiology.

Previous studies have focused on the role of HIF-1 in the development and progression of cancer. These studies have found an association with high levels of HIF-1 and poor cancer prognosis (Sivridis et al., 2002, Krishnamachary et al., 2003, Baba et al.). Induction of HIF-1 in the hypoxic environment of the tumour is thought to aid proliferation and angiogenesis. This exaggerated physiological response is associated with disease progression. High levels of HIF-1α have also been observed in ectopic endometrial deposits in the myometrial layer of women with adenomyosis, when compared to eutopic endometrium in the same subjects (Goteri et al., 2009). This increase in HIF-1α was associated with higher levels of VEGF and increased microvascular density, leading the authors to propose that increased levels of HIF-1α were involved in the pathogenesis of this condition.

Unlike cancer and adenomyosis, where an exaggerated physiological response contributes to disease, heavy menstrual bleeding may result from a defective response. Much of the knowledge regarding the physiological role of HIF-1 *in vivo* has been generated using knockout animals. Targeted inactivation of HIF-1α or HIF-1β in the mouse resulted in embryonic lethality due to vascular abnormalities (Iyer et al., 1998, Ryan et al., 1998, Maltepe et al., 1997). To overcome lethality and study the effects of HIF-1 in specific tissue sites, Cre recombinase-loxP technology has been used. These studies have demonstrated that HIF-1α not only mediates tissue adaptation to hypoxia but also has a fundamental role in physiological function. Utilising conditional HIF-1α knockouts in the mouse, HIF-1α has been shown to be necessary for the infiltration and activation of myeloid cells (Cramer et al., 2003). The resulting metabolic defect of HIF-1α deficiency is a profound impairment of innate immunity. In the gastrointestinal tract, HIF-1α also appears to maintain colonic barrier protection during hypoxic and inflammatory insults (Furuta et al., 2001, Karhausen et al., 2004), suggesting a potential therapeutic role for HIF-1α in inflammatory conditions such as colitis. The finding of reduced HIF-1α in the menstrual endometrium of women with HMB is therefore consistent with a defective physiological response. Endometrial inflammation and tissue destruction leads to

menstrual bleeding. Decreased levels of endometrial HIF-1α at this time may contribute to excessive tissue damage, defective repair and subsequent HMB.

To further examine the contribution of HIF-1 to endometrial repair, the expression of VEGF, a known target of HIF-1, was determined in the menstrual endometrium of women with NMB and HMB. VEGF is a potent angiogenic factor that stimulates endothelial cell proliferation and migration (Ferrara, 2004). Therefore, it has an attractive role in the repair of damaged blood vessels at menstruation. A recent study of the decidualised mouse uterus and rhesus macaque endometrium, found that VEGF blockade with VEGF Trap completely inhibited neovascularisation and reepithelialisation during endometrial repair (Fan et al., 2008). Data presented in this chapter clearly demonstrate aberrant endometrial expression of VEGF in women with HMB. There were significantly lower levels of VEGF mRNA and protein in the menstrual endometrium of women with HMB when compared to normal controls. These findings are in agreement with a previous study, which found VEGF mRNA and protein were significantly reduced in menstrual effluent from women with HMB when compared to normal controls (Malik et al., 2006). The finding of increased VEGF receptor expression during the late secretory and menstrual phases of the cycle is in agreement with a previous study (Nayak et al., 2000) and suggests it may be regulated by progesterone withdrawal. In addition to significantly decreased VEGF levels in the menstrual endometrium of women with HMB, another downstream target of HIF-1 was also shown to be aberrantly expressed. CXCR4 mRNA was significantly decreased in the menstrual endometrium of women with HMB versus controls, in a pattern similar to that of VEGF and HIF-1.

To examine the functional consequences of decreased endometrial HIF-1, an *in vitro* tube formation assay was performed using conditioned media from endometrial epithelial cells. Cells in which HIF-1α had been silenced using ShRNA produced a significantly decreased angiogenic response compared to untransfected cells. This finding confirms that endometrial HIF-1α has the ability to induce a significant angiogenic response. This response is likely to be due to induction of a number of angiogenic factors, including VEGF, AM, IL-8 and CTGF (Forsythe et al., 1996,

Cormier-Regard et al., 1998, Kim et al., 2006, Higgins et al., 2004). Replacement of VEGF in the HIF-1α silenced conditioned media, only partially restored the angiogenic response. Therefore, treatments to correct the endometrial defect in women with HMB may be most effective when they replace multiple angiogenic factors.

Prolyl hydroxylase inhibitors may provide an effective therapeutic intervention for women with HMB. They prevent hydroxylation of proline residues on the HIF-1α subunit, stabilising HIF-1α in normoxic conditions and thereby increasing transcription of target genes. PHD inhibitors have been shown to protect mice and rats from kidney ischaemia-reperfusion injury (Hill et al., 2008, Bernhardt et al., 2006) and have had beneficial effects in the murine model of colitis (Cummins et al., 2008, Robinson et al., 2008). As the endometrium displays similar features of inflammation and vasoconstriction, these treatments may have substantial beneficial effects in women with HMB. Moreover, the endometrium is amenable to intermittent, local administration of pharmacological treatments, allowing minimisation of potential side effects. Monitoring of the efficacy of these treatments may be possible with subjective pictorial menstrual charts, as results herein demonstrate a good correlation with objective measurements, in agreement with previous studies (Wyatt et al., 2001, Wilkens et al., 2008). Examination of the effects of compounds which stabilise HIF-1α in animal models of menstruation will further elicit their risks and benefits in the treatment of HMB.

6.4.1 Summary

The data presented in this chapter detail differences in the human endometrium of women with normal and heavy menstrual bleeding at the time of endometrial repair. Altered gene expression, lower levels of HIF-1α protein and an aberrant angiogenic response at this time may all contribute to the presentation of HMB. Further delineation of (i) the physiological processes at menstruation and (ii) aberrations that occur in the endometrium of women with HMB may promote significant advances in the development of novel treatments for these women.

7. General Discussion

7.1 Synopsis of Results

The data presented in this thesis describe the presence and regulation of known angiogenic and mitogenic factors in the human endometrium. Interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), adrenomedullin (AM), connective tissue growth factor (CTGF) and endothelin-1 (ET-1) were all found to be maximally expressed during the perimenstrual phase of the human menstrual cycle. The timing of this repair factor up-regulation is consistent with previous microscopic examination of the human endometrium, which revealed that endometrial repair commences on day two of the cycle (Ludwig and Spornitz, 1991). As oestrogen levels remain low during menstruation, it was hypothesised that factors involved in the initial endometrial repair process were regulated by declining progesterone levels following the regression of the corpus luteum.

Progesterone withdrawal is known to increase the levels of COX-2 enzyme in the endometrium, which subsequently increases prostaglandin synthesis (Critchley et al., 1999). In addition, there is evidence for a transient hypoxic episode in the uppermost endometrial layer as spiral arterioles constrict following progesterone withdrawal (Fan et al., 2008). Therefore, the effects of progesterone withdrawal, prostaglandin E_2, prostaglandin $F_{2\alpha}$ and hypoxic conditions on endometrial repair factor expression were examined. Results from *in vitro, ex vivo* and *in vivo* models of the perimenstrual phase revealed that progesterone withdrawal and the downstream induction of hypoxic conditions and prostaglandin production were all necessary to increase IL-8, AM and VEGF mRNA in endometrial tissue.

The mechanisms by which hypoxic conditions and prostaglandins increase these endometrial repair factors were then examined. The transcription factor hypoxia-inducible factor (HIF) had previously been shown to regulate IL-8, AM and VEGF in other tissue sites (Kim et al., 2006, Cormier-Regard et al., 1998, Forsythe et al., 1996) but the endometrial regulation of these factors by HIF had not been examined. Therefore, the presence of HIF-1α in the human endometrium was examined in tissue from across the menstrual cycle. Western blotting for HIF-1α revealed a transient presence of HIF-1α, with stable protein found only during the perimenstrual

phase. To examine the contribution of HIF-1α to repair, pharmacological inhibition of HIF-1 binding and silencing of HIF-1α using RNA interference was performed in endometrial cells. Data from these experiments suggested that two pathways of repair factor induction are present in the perimenstrual endometrium; a HIF-1α dependent hypoxic pathway and prostaglandin mediated induction that is independent of HIF-1α (Figure 111).

NF-κB is also a transcription factor involved in the up-regulation of a wide variety of genes, including IL-8 (Kayisli et al., 2004). NF-κB has been demonstrated in human endometrial biopsies during the perimenstrual phase (King et al., 2001). Progesterone has inhibitory effects on NF-κB activity, suppressing its activity until menstruation is required (Kelly et al., 2001). Therefore, its role in the induction of endometrial repair factors downstream of progesterone withdrawal was examined using a dominant negative inhibitor of NF-κB. Initial experiments demonstrated that this transcription factor may contribute to the normoxic induction of IL-8 by PGE_2 and suggested interactions with HIF-1α could enable synergistic increases in IL-8 when both hypoxic conditions and PGE_2 are present simultaneously (Figure 111).

Finally, to assess the possibility that suboptimal endometrial repair contributes to the pathogenesis of heavy menstrual bleeding (HMB), an unbiased whole genome array was carried out to compare gene expression in menstrual endometrium from women with objectively measured heavy and normal menstrual blood loss. This revealed 259 differentially expressed genes between the two groups. Differential expression of five genes was confirmed by Q-RT-PCR (Figures 92, 93, 94, 96, 97). Functional analysis of the differentially expressed gene set determined that the most relevant network processes included response to steroid hormone stimulus, regulation of cell cycle and cell communication and signalling. Of the 171 genes that were down-regulated in women with HMB versus normal controls, CXCR4 was the most significantly decreased gene. Interestingly, CXCR4 is a known target of HIF-1 (Ishikawa et al., 2009, Pan et al., 2006). Therefore, the levels of HIF-1α were assessed in the menstrual endometrium of four women with HMB and four normal controls. Densitometric analysis of Western blotting for HIF-1α demonstrated that

women with HMB had significantly less HIF-1α protein than controls. Moreover, women with HMB had significantly reduced menstrual CXCR4 and VEGF mRNA expression than women with normal loss. Decreased expression of these known target genes of HIF-1 is consistent with Western blot data and suggests the HIF-1 system may be aberrant in women with HMB. This may be due to a defective hypoxic response secondary to inefficient spiral arteriole vasoconstriction (discussed in Chapter 1.6.3.1). This hypothesis concurs with previous studies detailing reduced levels of the potent vasoconstrictor endothelin-1 and a reduced $PGF_{2\alpha}$:PGE_2 ratio in the endometrium of women with HMB versus normal controls (Marsh et al., 1997, Smith et al., 1981a). Alternatively, normoxic induction of HIF-1α may be aberrant in the endometrium of women with HMB. Although the data herein demonstrate HIF-1α was not induced by prostaglandins in normoxia, the effects of other inflammatory mediators were not examined. Furthermore, the endometrial response to progesterone withdrawal remains to be determined in women with heavy and normal loss. Women with HMB may have delayed or defective progesterone withdrawal during the perimenstrual period, resulting in aberrant endometrial repair.

To determine the functional impact of decreased endometrial HIF-1α protein levels, HIF-1α was silenced in endometrial cells with RNA interference. The angiogenic potential of the culture supernatant of these HIF-1α depleted cells was then compared to that of untransfected endometrial cells. This revealed that decreased endometrial HIF-1α levels resulted in a significantly reduced angiogenic response. Therefore, reduced endometrial HIF-1α *in vivo* may delay the repair of blood vessels and result in women experiencing prolonged, heavy menstrual bleeding (Figure 112).

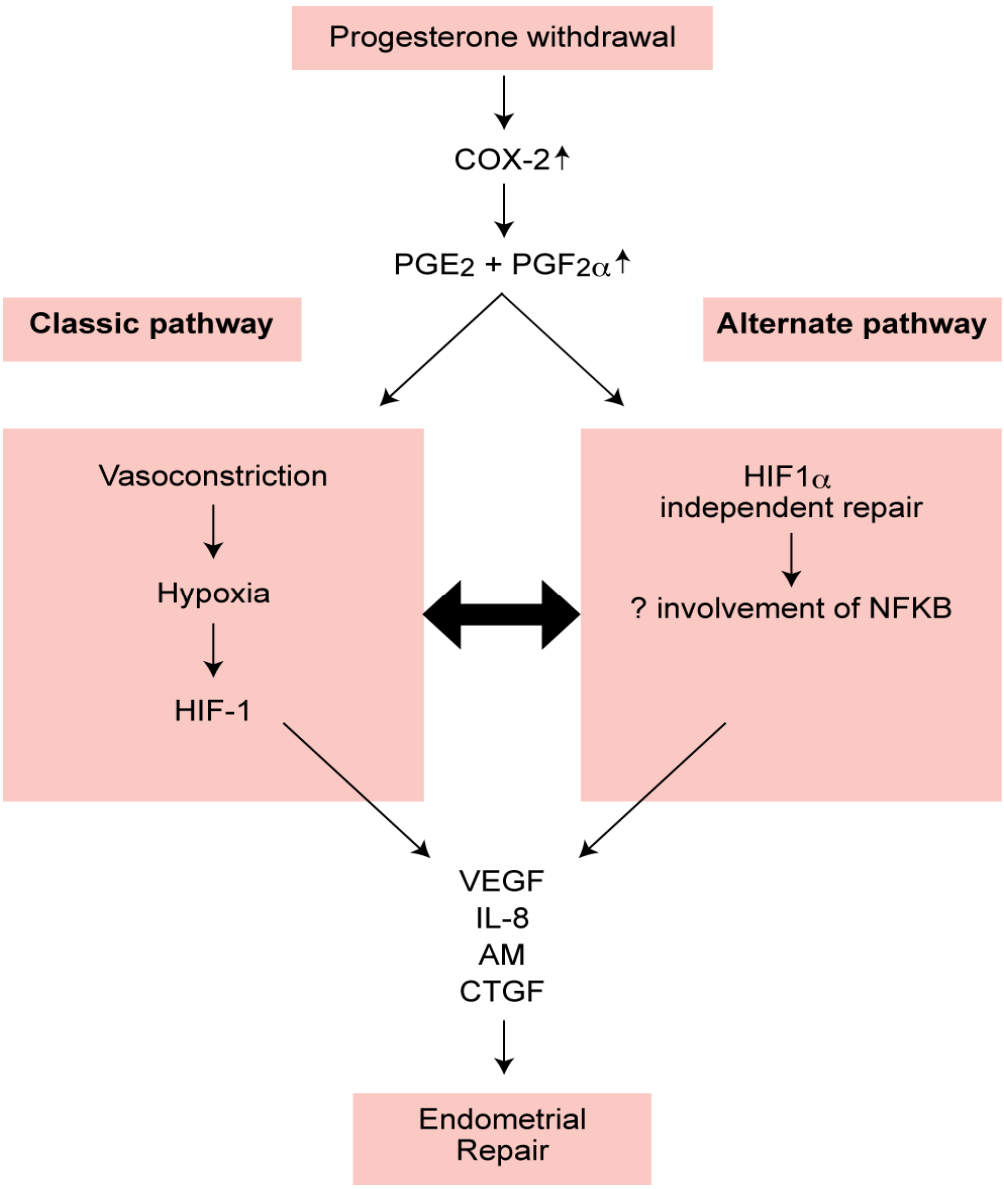


Figure 111. **The two pathway hypothesis of endometrial repair factor induction at menstruation.** Following progesterone withdrawal in the late secretory phase, endometrial cyclooxygenase-2 (COX-2) levels are increased. This results in the synthesis of prostaglandins (PG). $PGF_{2\alpha}$ is a potent vasoconstrictor and, in collaboration with other factors, causes vasoconstriction of the spiral arterioles. The subsequent hypoxic insult stabilises endometrial hypoxia inducible factor-1α (HIF-1α) to allow increased transcription of endometrial repair factors such as interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), adrenomedullin (AM) and connective tissue growth factor (CTGF). An alternative pathway for repair factor induction also exists, where endometrial PGs stimulate HIF-1α independent increases in repair factor expression, perhaps via nuclear factor kappa B (NFκB). Results of this thesis suggest that there may be some interaction between these two pathways to induce synergistic increases in factors involved in endometrial repair.

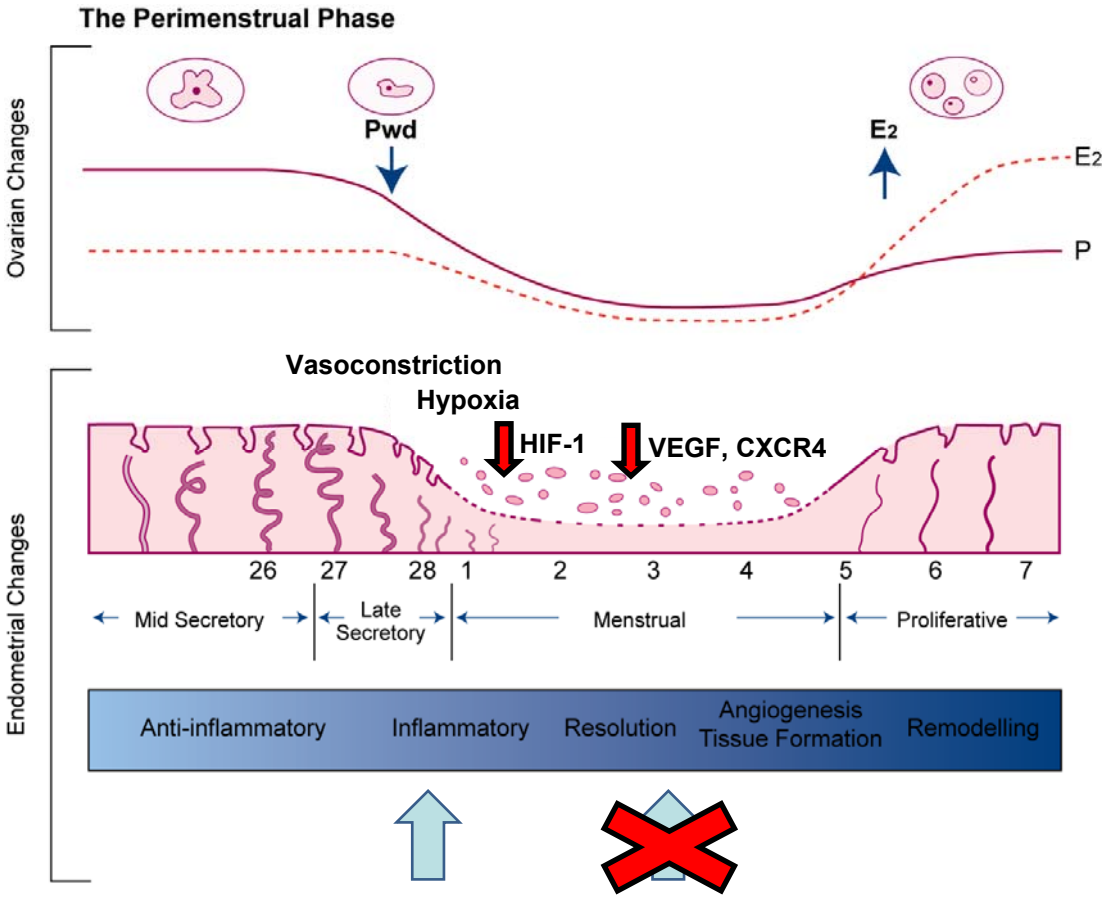


Figure 112. **Endometrial changes during the perimenstrual phase and aberrations in women with heavy menstrual bleeding (HMB).** Following progesterone withdrawal in the late secretory phase, there is vasoconstriction of the endometrial spiral arterioles. This leads to a transient hypoxic insult in the uppermost endometrial zones. Hypoxia inducible factor (HIF)-1α is stabilised in the human endometrium, leading to induction of endometrial repair factors, such as vascular endothelial growth factor (VEGF) and CXCR4. Women with HMB have decreased endometrial HIF-1α protein levels at menstruation and decreased VEGF and CXCR4 expression. Delayed endometrial repair secondary to these aberrations may contribute to their heavy and prolonged menstrual blood loss.

7.2 Areas for future study

1. This thesis details the endometrial expression of IL-8, AM, VEGF, CTGF and ET-1 across the menstrual cycle. Results demonstrate that these factors are selectively increased in the perimenstrual phase. Limited analysis of the functional consequence of these changes was assessed with an *in vitro* angiogenesis assay. Further analysis of other aspects of endometrial repair, such as cell proliferation, wound healing and apoptosis, would be valuable to compare the functional potential of the endometrium at different stages of the cycle. Development of *in vitro* assays to compare culture supernatants of proliferative, secretory and menstrual explants with and without antibody neutralisation of specific repair factors will further delineate the mechanisms of endometrial repair.

2. The study of multiple endometrial biopsies from the same woman at various stages of her menstrual cycle would eliminate patient variability and allow paired analysis of repair factor expression. Unfortunately ethical approval was not obtained for collecting multiple endometrial samples in this study but should be considered in future ethical applications.

3. Data displayed in chapter 4 of this thesis demonstrate that PGE_2 and $PGF_{2\alpha}$ have a significant impact on endometrial repair factors in endometrial cells and endometrial tissue explants. The role of PGI was not studied, although a contribution to endometrial function has previously been identified (Battersby et al., 2004, Smith et al., 2006, Smith et al., 1981b). Examination of the impact of PGI on endometrial repair factors is necessary to fully elucidate the contribution of prostaglandins to the repair process.

4. This thesis contains the novel findings of (i) selective HIF-1α stabilisation in the perimenstrual endometrium and (ii) significantly decreased HIF-1α levels in women with heavy versus normal bleeding. Future assessment of

endometrial levels of the alternative alpha subunits, HIF-2α and HIF-3α, is necessary to delineate the role of HIF in the human endometrium.

5. This work demonstrates that prostaglandins act independently of HIF-1α to increase endometrial repair factor expression. However, the contribution of HIF-1α to the production of prostaglandins was not assessed. It would be interesting to determine the effect of hypoxia and HIF-1α on endometrial COX-2 levels. In addition, the contribution of NF-κB to endometrial repair factor expression deserves further examination. The effect of inhibition of NF-κB on PGE_2 induced IL-8 expression was examined but the contribution of NF-κB to $PGF_{2\alpha}$ mediated increases was not assessed. Furthermore, the interactions between HIF-1α and NF-κB in the human endometrium provide an attractive area of future research.

6. The whole genome array study of menstrual endometrium from women with heavy and normal bleeding requires further validation with Q-RT-PCR and by confirmation of protein levels. In addition, the *in silico* analysis of the differentially expressed gene group would be enhanced with *in vitro* functional studies of menstrual endometrium to confirm differences in apoptosis, angiogenesis or proliferation between women with heavy and normal menstrual bleeding.

7. Herein, women with HMB were shown to have significantly reduced HIF-1α protein levels and defective expression of downstream VEGF and CXCR4 mRNA. It remains to be determined if this is a result of defective vasoconstriction and prevention of a hypoxic stimulus. With advancements in magnetic resonance imaging, it may be possible to measure the endometrial perfusion of women with objective blood loss measurements during the immediate pre-menstrual phase. Furthermore, when the safety profile of pimonidazole is determined, this marker of hypoxia could be administered to women prior to menstrual endometrial sampling to ascertain any differences in expression between women with heavy and normal menstrual blood loss.

8. Genetic disorders can result in stabilisation of HIF-1α in normoxic conditions. Von Hippel-Lindau disease is a result of a mutation in the Von Hippel-Lindau protein, which is involved in targeting HIF-1α for proteosomal degradation (Semenza, 2000). Individuals with mutations in prolyl hydroxylase domains have also been described (Percy et al., 2006). Individuals with these conditions over-express HIF-1α protein. Despite the systemic nature of these conditions, determination of the menstrual blood loss of women with these disorders may provide interesting insights into the contribution of HIF-1 to endometrial physiology.

7.3 Conclusions

The data contained in this thesis support the role of progesterone withdrawal, hypoxia and prostaglandins in the regulation of endometrial repair factors at menstruation. Delineation of the regulation of repetitive endometrial injury and its efficient repair may have important translational implications for other tissue sites. The endometrium heals without scarring or loss of function and further research into these processes may reveal novel therapeutic targets for conditions such as persistent inflammation and problematic scarring.

In addition, this thesis details significant differences in gene expression in the menstrual endometrium of women with normal and heavy menstrual bleeding. A potential pathway involving reduced menstrual levels of HIF-1α and its downstream transcription targets was identified in women with objectively measured HMB, consistent with suboptimal repair in these women. Compounds that stabilise HIF-1α may help restore efficient endometrial repair. Such treatments could be delivered locally during the menstrual phase to limit potential side effects. Continued research to introduce an effective medical treatment for women with HMB will allow significant improvements in quality of life for many women and lead to widespread socio-economic benefits.

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